Anita da Silva Lourenço

Licenciada em Bioquímica

Improved polymeric medical devices for active substances delivery

Dissertação para obtenção do Grau de Mestre em Biotecnologia

Orientador: Dr. Teresa Casimiro, FCT-UNL

Co-orientadores: Dr. António Soares, BeyonDevices, Lda

Prof. Dr. Ana Aguiar-Ricardo, FCT-UNL

Júri:

Presidente: Prof. Dr. Pedro Simões

Arguente: Dr. Ana Matias

Vogais: Dr. Teresa Casimiro

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro, 2014

Improved polymeric medical devices for active substances delivery

Acknowledgments

I would like to express my sincere gratitude to many people that spent this last year with me and helped me in this final phase of my master's graduation.

My first acknowledgement is for Dr. Teresa Casimiro, for all the commitment, guidance, support, constructive advices and incentives. There is no words to express my gratitude for all the support during the time that I spent in this laboratory.

I also wish to thank to Prof. Ana Aguiar-Ricardo, my co-supervisor, for always challenging me to go further, for the interesting suggestions and for the precious contribution in the development of this thesis.

Thank you both for kindly hosted me in your lab, it was a great pleasure to work with you!

I want to thank also to Dr. António Soares, for accepting my proposal to work with us, for the availability to co-supervise my thesis and for all the support as intermediary between the university and the company.

To BeyonDevices, for giving me the opportunity to develop my thesis in a collaboration university-company and for kindly provide me the material that I needed for the development of this project.

Funding from Fundação para a Ciência e Tecnologia (FC&T-Lisbon) through contracts PEst-C/EQB/LA0006/2013 and PTDC/EQU/116097/2009 is acknowledged.

To Prof. Ilda Sanches and Dr. Rosario Mato, my very special thank for receiving me at your lab at DCV, for providing me a 'microbial part' in this work and for giving me all the support I needed during its development.

To Prof. Alexandra Fernandes and Pedro Martins, for analyzing my samples and for all the extra-h spent with my work.

To Prof. Pedro Simões and Pedro Lisboa, that gently gave me the lavender oil.

To Prof. Ana Rego, for the XPS assays.

To Mrs. Maria José Carapinha, Mrs. Conceição and Mrs. Idalina for all the help.

Further, I would like to thank to 510 lab team, Dr. Vasco Bonifácio, Rita Restani, Sofia Silva, Telma Barroso, Renato Cabral, Tiago Reis, Rita Pires, José Pinto and Raquel Viveiros. A special thanks to Vanessa Correia and Patrícia Morgado, for all the help, suggestions, discussions and friendship. You are the best team work!

To my dear master colleague and friend Márcia Tavares, for the support, friendship and all the good moments.

And finally, I would like to thank to my parents, the most important people of my life without whom this was not possible. Thank you for always believing in me. You are the best!

Also to all my family and friends, for all the care and support. Thank you!

Abstract

The work presented in this thesis was developed in collaboration with a Portuguese company, BeyonDevices, devoted to pharmaceutical packaging, medical technology and device industry. Specifically, the composition impact and surface modification of two polymeric medical devices from the company were studied: inhalers and vaginal applicators.

The polyethylene-based vaginal applicator was modified using supercritical fluid technology to acquire self-cleaning properties and prevent the transport of bacteria and yeasts to vaginal flora. For that, *in-situ* polymerization of 2-substituted oxazolines was performed within the polyethylene matrix using supercritical carbon dioxide. The cationic ring-opening polymerization process was followed by end-capping with *N,N*-dimethyldodecylamine. Furthermore, for the same propose, the polyethylene matrix was impregnated with lavender oil in supercritical medium.

The obtained materials were characterized physical and morphologically and the antimicrobial activity against bacteria and yeasts was accessed. Materials modified using 2-substituted oxazolines showed an effective killing ability for all the tested microorganisms, while the materials modified with lavender oil did not show antimicrobial activity. Only materials modified with oligo(2-ethyl-2-oxazoline) maintain the activity during the long term stability. Furthermore, the cytotoxicity of the materials was tested, confirming their biocompatibilty.

Regarding the inhaler, its surface was modified in order to improve powder flowability and consequently, to reduce powder retention in the inhaler´s nozzle. New dry powder inhalers (DPIs), with different needle's diameters, were evaluated in terms of internal resistance and uniformity of the emitted dose. It was observed that they present a mean resistance of 0.06 $cmH_2O^{0.5}$/(L/min) and the maximum emitted dose obtained was 68.9% for the inhaler with higher needle´s diameter (2 mm). Thus, this inhaler was used as a test and modified by the coating with a commonly-used force control agent, magnesium stearate, dried with supercritical carbon dioxide ($scCO_2$) and the uniformity of delivered dose tests were repeated. The modified inhaler showed an increase in emitted dose from 68.9% to 71.3% for lactose and from 30.0% to 33.7% for Foradil.

Key-words: Polyethylene, oligo(2-oxazoline)s, supercritical CO_2, lavender oil, antimicrobial activity, self-disinfecting, dry powder inhaler, internal resistance, emitted dose, surface modification, powder retention, force control agent.

Resumo

O trabalho apresentado nesta tese foi desenvolvido em colaboração com uma empresa de Portuguesa, BeyonDevices, dedicada à produção de embalagens farmacêuticas, tecnologia médica e indústria de dispositivos. Especificamente, estudou-se o efeito da modificação de superfície e composição de dois dispositivos médicos poliméricos da empresa: aplicadores vaginais e inaladores.

O aplicador vaginal de polietileno de alta densidade foi modificado utilizando tecnologia de fluídos supercríticos para adquirir propriedades de auto-limpeza e evitar o transporte de bactérias e fungos para a flora vaginal. Para isso, foi realizada uma polimerização *in-situ* de 2-oxazolinas na matriz do polietileno, utilizando dióxido de carbono supercrítico. O processo de polimerização catiónica de abertura de anel foi seguido por uma terminação usando *N,N*-dimetildodecilamina. Além disso, para o mesmo propósito, a matriz de polietileno foi impregnada com óleo de alfazema em meio supercrítico.

Os materiais obtidos foram caracterizados física e morfologicamente, e a atividade antimicrobiana contra bactérias e fungos foi avaliada. Materiais modificados usando oxazolinas-2-subtituída mostraram-se efetivos na morte de todos os microrganismos testados, enquanto os materiais modificados com óleo de alfazema não apresentaram atividade antimicrobiana. Apenas os materiais modificados com oligo(2-etil-2-oxazolina) mantêm a atividade durante os testes de estabilidade a longo prazo. Além disso, estudou-se a citotoxicidade dos materiais, confirmando que todos são biocompatíveis.

Em relação ao inalador, a sua superfície foi modificada com o objetivo de melhorar a fluidez do pó e, consequentemente, reduzir a retenção de pó no bocal dos inaladores. Avaliaram-se os novos inaladores de pó seco (DPIs), com diferentes diâmetros de agulhas, em termos de resistência interna e uniformidade da dose emitida. Concluiu-se que eles apresentam uma resistência média de 0,06 $cmH_2O^{0.5}/(L/min)$ e que a máxima dose emitida foi de 68,9% para o inalador com maior diâmetro de agulha (2 mm). Assim, este inalador foi usado como teste e a superfície modificada com um agente de controlo da força vulgarmente utilizado, estearato de magnésio. Secou-se com dióxido de carbono supercrítico e repetiram-se estudos de dose emitida. O inalador modificado mostrou um aumento na dose emitida de 68,9% para 71,3% para a lactose e de 30,0% para 33,7% para Foradil.

Palavras-chave: Polietileno, oligo(2-oxazolina)s, CO_2 supercrítico, óleo de alfazema, atividade antimicrobiana, auto-desinfecção, inaladores de pó seco, resistência interna, dose emitida, modificação de superfície, retenção de pó, agentes de controlo de forças.

Abbreviation list

$BF_3.Et_2O$ – Boron trifluoride diethyl etherate

C. albicans – *Candida albicans*

CFU – Colony forming cells

C. glabrata – *Candida glabrata*

CROP – Cationic ring-opening polymerization

DPI – Dry poder inhaler

DSC – Differential scanning calorimetry

DUSA – Dose uniformity sampling apparatus

E. coli – *Escherichia coli*

EtOx – Oligo(2-ethyl-oxazoline)

MeOx – Oligo(2-methyl-oxazoline)

MgSt – Magnesium stearate

MHA – Muller-Hinton agar

MHB – Muller-Hinton broth

PE – Polyethylene

POx – Polyoxazolines

S. aureus – *Staphylococcus aureus*

SEM – Scanning electron microscopy

$scCO_2$ – Supercritical carbon dioxide

SCFs – Supercritical fluids

XPS – X-ray photoelectron spectroscopy

YMB – Yeast mannitol broth

Contents

Index of Figures

Chapter 1

Chapter 2

Index of Tables

Chapter 1

Chapter 2

Chapter 1: Antimicrobial self-clean of a Vaginal Applicator

1.1. Introduction

This chapter describes the adaptation strategy of this thesis for modification of the surface of a vaginal applicator in order to induce self-disinfecting ability, reduce the contamination and consequently the risk of bacterial and fungal infection during the handling of the vaginal applicator.

1.1.1. Medical devices

The use of medical devices is one of the biggest growing areas of medicine and an increasing source of healthcare associated infections (HAIs).[1]
Medical device infections are typically related with microbe colonization in devices and this contamination mostly happens by inoculation with only a few microorganisms from the consumer's skin or mucous during use.[2]
The ability to adhere to materials and promote formation of a biofilm is an important feature of the pathogenicity of microorganism involved in contamination. Biofilm formation occurs in two phases: a rapid attachment of the microorganism to the surface and a prolonged accumulation stage that involves cell proliferation and intracellular adhesion. Factors involved in microbial adherence include physicochemical forces as polarity, London-van der Waal's forces and hydrophobic interactions.[3] Once adhered to the surface, microorganisms multiply and accumulate in multilayered cell clusters, which requires intercellular adhesion, chemical interactions or quorum sensing mechanisms.[4]
Therefore, improving the bacterial resistance of polymer surfaces is a major goal in the development of the medical devices. In order to reduce the frequency and level of contamination of surfaces, the use of self-disinfecting surfaces is a promising method.[5]

1.1.2. Vaginal applicators

Intravaginal drug delivery has been conventionally restricted to the delivery of antinfectives to the local vaginal cavity. The vaginal route is a potential way for the delivery of therapeutically biological molecules, vaccines and hormones, mostly in the field of microbicides, mucosal vaccines and therapeutic proteins and peptides.

Applicators are planned to be introduced in the vagina, effectively deliver the therapeutic product, and then be removed (see Figure 1.1).[6]

Figure 1.1 ***BeyonDevices' Vaginal Applicator.***[6]

The healthy human vagina is dominated by lactobacilli, which plays an important role in protecting the host against urogenital infections. Vulvovaginal candidiasis (VVC) is an infection of the female genital tract caused by the abnormal growth of yeast-like yeasts. VVC is caused mainly by the genus *Candida*, where 80–90% of cases are due to *Candida albicans* and 10–20% due to the other species called not-*C. albicans* (*Candida tropicalis, Candida glabrata, Candida krusei, Candida parapsilosis, Candida pseudotropicalis, and Candida lusitaniae*). *C. glabrata* is the second specie most frequent in the VVC. The manifestations of VVC are often painful and uncomfortable and by affecting millions of women annually, being considered an important global public health problem.[7]

Urinary tract infections (UTIs) are one of the most frequent clinical bacterial contaminations in women, accounting for nearly 25% of all infections. Around 50–60% of women will develop UTIs in their lifetimes and *Escherichia coli* is the organism that causes UTIs in most patients. Despite the fact that most *E. coli* is eradicated by the host defense mechanisms within days, only small clusters of intracellular *E. coli* are observed to persist for several months in an antibiotic-resistant state.[8]

The self-disinfecting surface of a vaginal applicator can promote the reduction of the risk of infections caused by spread of microorganisms from external environment in vaginal flora.

1.1.3. Self-disinfecting surfaces

Innovative technologies have identified new approaches to develop self-disinfecting surfaces to minimize HAIs. These include changing the structure or surface to minimize the attachment of microbes or to delay the development of biofilms. The surface modification with long-term or even permanent antimicrobial activity is one key strategy for the provision of improved hygienic living conditions.

One of the strategies used for surface modification is to increase the surface hydrophilicity by grafting polar and hydrophilic groups such as alcohols or ethers and this can be performed by plasma treatment[9] and/or by grafting hydrophilic polymers.[10]

Another strategy consists in the impregnation or coating the surface with heavy metals (eg, silver or copper), however the poor biocompatibility of the agents (for example silver ions) is a challenge in the design and development of bactericide surfaces.[11,12]

Immobilization or impregnation of low molecular weight antibiotics, cationic antimicrobial peptides, lysozyme, chitosan and charged polymers on different surfaces has also been tested in the development of bactericidal surfaces. The use of antibiotics such as penicillin, ampicillin or gentamycin has provided long-term antimicrobial activity and low risk of side effects, but inducing bacterial resistance is the downside of this strategy.[13]

Antimicrobial peptides (AMPs) are short natural or synthetic peptides which consist of less than 50 amino acids, mostly with overall positive charge, and the mechanism to kill bacteria is mainly through a cell membrane disruption. The study and use of AMPs is increasing due to their broad spectrum antimicrobial activity, speed of action and low propensity for developing resistant mutants owing to their mechanism of bacteria killing.[14]

Antimicrobial properties are achieved by the immobilization of AMPs to polymer, however the conjugation of AMPs may compromise their anti-biofouling properties, once a decrease in peptide concentration resulted in an increased non-fouling character with a decrease of bactericidal activity, may lead to their poor long-term performance.[15] The development of bactericidal surfaces with lysozyme and chitosan is valuable and emerging, as natural agents are environmentally benign, but the bland bactericidal activity of chitosan and its influence on pH may limit its application.[16] Charged polymers, particularly quaternary ammonium polymers, can make potent antimicrobial coatings, however their toxicity and biocompatibility may limit their applications in materials for biomedical use.[17]

Generally, the death of the bacteria is a result of either bacterial membrane disruption or of a specific interaction of the immobilized antibacterial agent with a target biomolecule at the surface of the bacteria.

1.1.4. Antimicrobial polymers

Antimicrobial polymers are promising substitutes of low-molecular-weight antibiotics, environmentally problematic biocides and disinfectants. The majority of antimicrobial polymers are amphiphilic polycations, containing quaternary ammonium, phosphonium, or thertiary sulfonium groups. These macromolecules can be classified as biocide-releasing polymers, polymeric biocides and biocidal polymers.[18] Macromolecules belonging to first two classes are considered biodegradable and mostly release biocides that will destroy the vaginal flora. In opposition, biocidal polymers act as one molecule that is able to destabilize and eventually destroy the cell membrane of microorganisms resulting in cell lysis and cell death.[19] Even antibiotic-resistant bacteria such as methicillin-resistance *Staphylococcus aureus* (MRSA) can be killed effectively. Some examples of potentially biodegradable biocidal polymers have been described in the past few years.[20] An alternative to the traditional polymers are biocides coupled as end groups to inactive polymers.[21] If the polymer contains a second, nonantimicrobially active end group mentioned to as a satellite group, the antimicrobial activity of these polymers can be controlled. It was shown recently that polyoxazolines with an antimicrobial group are antimicrobially active.[22]

1.1.5. Poly(2-oxazoline)s

Poly(2-oxazoline)s (POx) constitute a polymer class with exceptional properties and great versatility with use in a wide range of biomedical applications and have been proposed as a versatile platform for the development of new medicines.[23] POx is a neutral hydrophilic polymer with non-fouling properties studied for inhibiting bacterial adhesion.[24]

Polyethylene glycol (PEG) is the most extensive used polymer in biomedicine, being used mainly to increase half-life and immunogenicity of proteins.[25] Although PEG remains the gold-standard in polymer based biomedical applications, based on its low polydispersity, biocompatibility and stealth behavior, it has some disadvantages and limitations. The polyether backbone of PEG is susceptible to oxidative degradation representing strong drawbacks particularly for long term applications as antifouling surfaces for implants, as well as possible induction of PEG-mediated complement activation has been recently reported.[26]

Thus, POx constitutes a probable candidate to overcome the drawbacks of PEG, retaining the required features for a polymer used in biomedical applications, such as

biocompatibility, stealth behavior, low dispersity, responsiveness, high functionalization possibilities, and high versatility attainable by copolymerization.[23] Poly(2-methyl-2-oxazoline) (PMeOx) and poly(2-ethyl-2-oxazoline) (PEtOx) have analogous stealth behavior as PEG while offering useful properties such as thermo-responsiveness, low viscosity and high stability.[27]

POx are readily obtained via the cationic ring-opening polymerization (CROP) of 2-oxazolines (see Figure 1.2). One huge advantage of the cationic ring-opening polymerization of 2-oxazoline monomers arises from the fact that they can be synthesized in a way that side-reactions such as the termination of chain growth and/or chain-coupling are suppressed (because of the control of this type of polymerization, it has been described as living polymerization).[28]

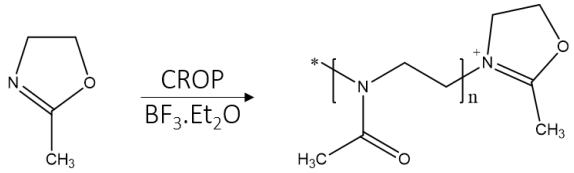


Figure 1.2 Reaction scheme to general strategy to synthesize 2-oxazolines (e.g. 2-methyloxazoline) by cationic ring-opening polymerization (CROP), yielding a living polymer.[29]

The *living polymers* of POx could be end-functionalized with various quaternary ammonium groups and the obtained oligomers exhibit antimicrobial activity due to this satellite groups. This strategy allow to obtain polymers with antimicrobial activity, low cytotoxicity, fast killing rate and broad-spectrum biocidal activity, representing a good approach to avoid and overcome bacterial resistance.[30]

1.1.6. Mechanism of action of end-capped poly(2-oxazoline)s

The biocidal mechanism, of such polymers is still not entirely clear. Such compounds have an antimicrobial action if they contain an alkyl chain of adequate length to interact with the bacterial membrane by binding to it. Then the membrane's equilibrium is disturbed due to the positive charge of the bioactive function eventually leading to cell death (see Figure 1.3).[31,32] POx derivatives only show bactericide activity if equipped with ammonium compounds that are active as low molecular weight pendants.

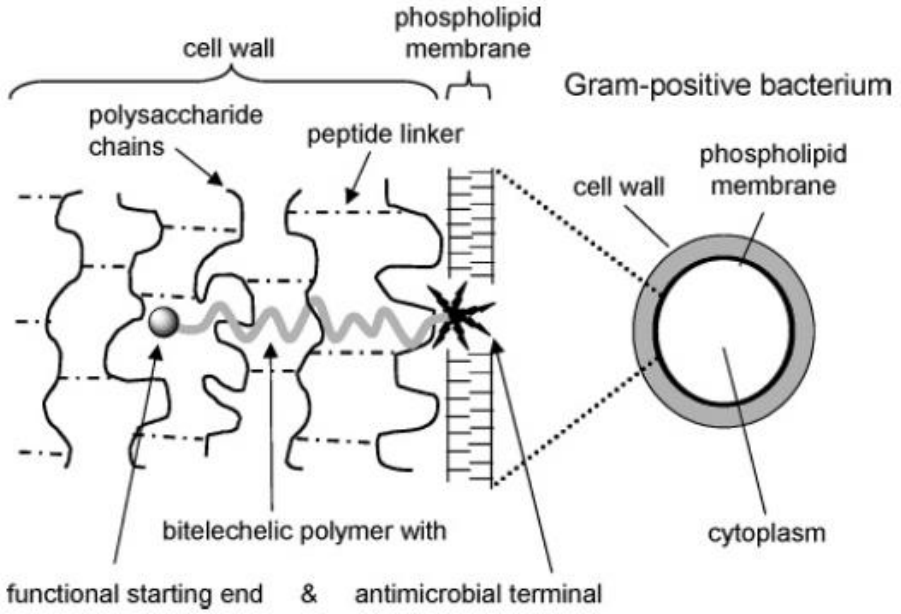


Figure 1.3 One proposed interaction between an antimicrobial polymer and a Gram-positive bacterium.[22]

1.1.7. Lavender oil

In addition to Pox, essential oils are well recognized in traditional medicine as antimicrobial agents and they are characterized by a wide spectrum of activity against bacteria and yeasts.

Lavender oil is well known for its application in aromatherapy, cosmetics, soaps and perfumes. Lavender essential oil is usually produced by steam distillation, from both the flower heads and foliage, but the chemical composition differs greatly, with the sweeter and most aromatic oil being derived from the flowers.[33] Today, the pure oil is most often used in aromatherapy or incorporated into soaps and other products as a pleasant fragrance or as an antimicrobial agent.

Lavender oil (primarily *Lavandula angustifolia*) has been found to be active against many species of bacteria and yeasts.[34] It has also been suggested that essential oils, including lavender, may be useful in treating bacterial infections that are resistant to antibiotics. For example, *L. angustifolia* oil has demonstrated to have *in vitro* activity against both MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant *enterococci*).[35]

Some cases of activity against bacteria and yeasts have been reported in literature, which incentive its application in antifouling and antimicrobial materials.[36]

1.1.8. Supercritical fluids (SCFs)

Supercritical fluids have gained interest as reaction solvent or processing agent. Recently, they have been applied in polymerization, swelling, impregnation, fractionation, purification and formation of powdered polymers. By definition, SCFs are substances at a temperature and pressure higher than their critical values, and which have a density close to or higher than its critical density (Figure 1.4 and Figure 1.5).[37] SCFs may be relatively dense and dissolve certain solids while being miscible with permanent gases and exhibiting high diffusivity and low viscosity. In addition, SCFs are highly compressible and the density dependent properties (and therefore solvent properties) can be “tuned” over a wide range by varying pressure.[38]

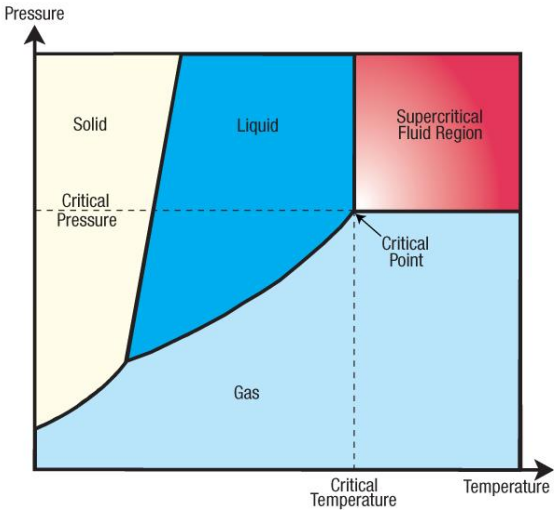


Figure 1.4 Schematic pressure-temperature phase diagram for a pure component showing the supercritical Fluid (SCF) region.[39]

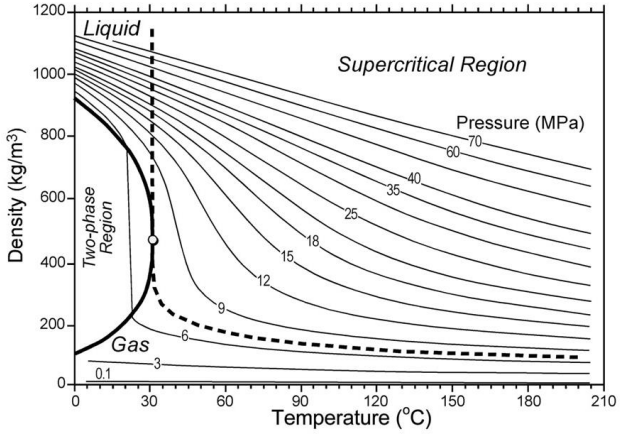


Figure 1.5 Carbon dioxide density-temperature phase diagram.[40]

In particular supercritical carbon dioxide ($scCO_2$) has been studied extensively for the synthesis and processing of polymers. CO_2 is naturally occurring, abundant and existing in natural reservoirs. In comparison to other substances, CO_2 has an easily accessible critical point with a critical temperature (Tc) of 31.1 °C and a critical pressure (pc) of 7.38 MPa. It is an ambient gas, and can be recycled after use. Finally, it is inexpensive and non-flammable.[41]

CO_2 can be separated from a reaction mixture by reducing the pressure, yielding a dry polymer product and in one-step process. It is possible the production of materials with high purity, since the final depressurization and extraction enables the removal of unreacted monomers and initiator from the polymer matrix. Also, it is possible to obtain polymers by a relatively simple and inexpensive process.

SCFs have also been recognized as excellent extraction solvent, due its availability and safety being considered as a GRAS (Generally Recognized as Safe) solvent. Such advantages make $scCO_2$ a solvent of choice in food and fragrances industry proven by coffee decaffeination, hop and essential oil extraction processes.[42]

1.1.9. $scCO_2$-assisted *in-situ* polymerization in polyethylene

Polyethylene (PE) is considered the most important and extensively used thermoplastic because of its low cost, good processability and several range of applications. However, PE has some disadvantages such as low surface energy, lack of chemical functionality, difficulty in dyeing and poor compatibility with synthetic polar polymers. The characteristics modification of PE has been widely investigated for electrical, coating, bonding, and biomedical applications.

The density of SCFs, and therefore their solvent strength, is tunable from gas to liquid by changing pressure and temperature. This offers the ability to control the degree of polymers' swelling, as well as the partitioning of small molecules penetrating between swollen polymer and fluid phases.[43] The low viscosity and almost inexistent surface tension of SCFs allow for rapid mass transfer into a swollen polymer.

Since the capacity of a solvent to dissolve solutes mainly depends on its density, changes in temperature or pressure may significantly change the dissolution properties of a supercritical solvent without variation of its composition. Additionally, the viscosity of $scCO_2$ is much lower than that of liquid solvents, and also varies strongly with deviations in pressure and temperature as stated before. Because of these features, diffusion coefficients of monomers and polymers are comparable to those of gases in SCFs. Any

small change in temperature or pressure, in particular near the critical point, has a large effect on the diffusivities.[44]

$ScCO_2$ has been used to impregnate polymers matrices with different additives. Using $scCO_2$ as a swelling agent, is possible to develop a synthetic method to produce new polymer composites.[45] Both the monomer and initiator are dissolved in $scCO_2$, impregnated into the polymer substrate, and subsequently polymerized (see Figure 1.6). Using this method, it is possible to obtain polymer composites, even when using polymers that are immiscible and cannot be obtained through conventional methods.

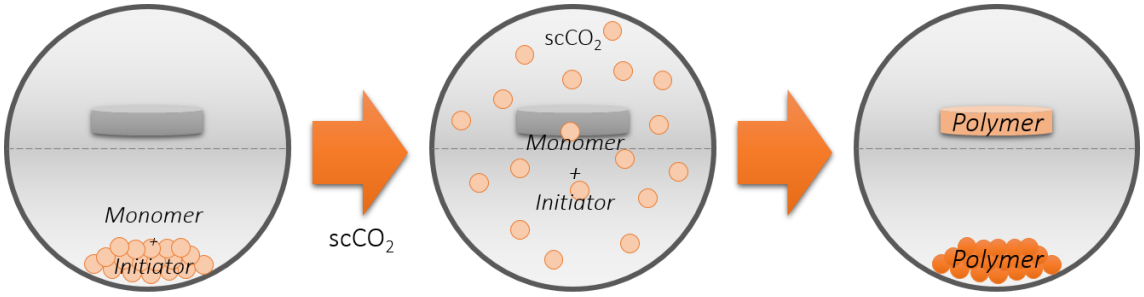


Figure 1.6 General representation for the in-situ polymerization in $scCO_2$. The monomer, initiator and impregnating-material are placed in a high-pressure cell and the CO_2 is added. A supercritical phase occurs, the monomer and initiator solubilize and the polymerization starts. After the end of polymerization, the non-impregnated polymer precipitates and CO_2 is released.

In this work, an *in-situ* polymerization was performed in order to synthesize oligo(2-oxazoline)s terminated with a quaternary ammonium inside the PE matrix, in $scCO_2$. During $scCO_2$ assisted process, PE swells and POx polymerizes inside the PE matrix. At the end of the procedure, the reactor is vented, PE returns to its initial form and trapps the antimicrobial POx inside its structure.[46]

1.1.10. $ScCO_2$-assisted impregnation

The polymer sorption and swelling processes in supercritical media and, in particular in $scCO_2$, became recently an area of increasing attention. Swelling and plasticization by $scCO_2$ sorption lead to the polymer transition from a glassy state into a rubbery state. In a rubbery state, the polymer chains can move more freely, which makes impurity extraction and polymer impregnation with additives more efficiently.

The impregnation process is feasible when the active substance (the solute) is soluble in the supercritical fluid, the polymer is swollen by the supercritical solution and the partition coefficient is favorable enough to allow the matrix to be charged with enough solute (see Figure 1.7).[47]

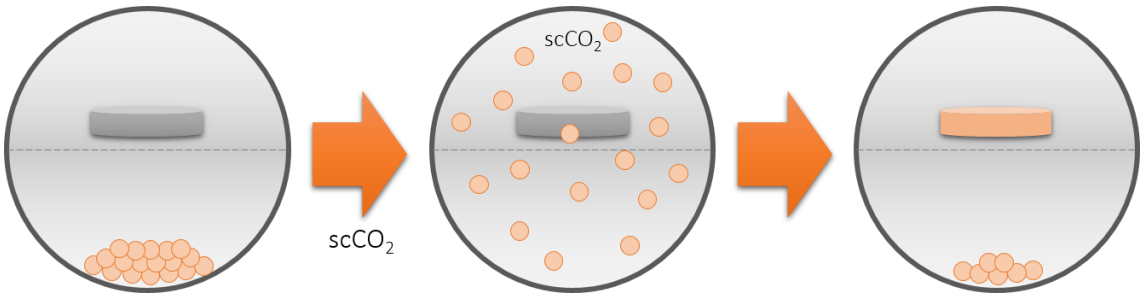


Figure 1.7 General representation for a $scCO_2$-assisted impregnation. The compound and impregnating-material are placed in a high-pressure cell and the CO_2 is added. A supercritical phase occurs, the compound solubilize and the impregnation starts. After depressurization, the non-impregnated polymer precipitates.

In this work, the lavender oil was impregnated in the PE matrix, using $scCO_2$, leading to a structure with antimicrobial properties and a characteristic fragrance.

1.2. Experimental

1.2.1. Materials

The high density polyethylene was supplied by BeyonDevices and used without further purification. The monomers 2-methyl-oxazoline and 2-ethyl-oxazoline, the initiator boron trifluoride diethyl etherate ($BF_3.Et_2O$) and *N,N*-dimethyldodecylamine were purchased from Sigma-Aldrich. All commercial reagents were used as received. Carbon dioxide was supplied by Air Liquide with a purity lighter than 99.998%. Dulbecco's modified Eagle's medium (DMEM-F12) and resazurin based *in vitro* toxicology assay kit were purchased from Sigma-Aldrich. Human fibroblast cells (Normal human dermal fibroblasts adult, cryopreserved cells) were purchased from PromoCell. Fetal bovine serum (FBS) was purchased from Biochrom AG.

1.2.2. Instrumentation

The thermal behavior of the samples was measured using Differential Scanning Calorimetry (DSC) in a Perkin Elmer DSC 7. The samples (~12mg) were analyzed under a flow of nitrogen gas and taken between 20^{o}C and 200^{o}C at a scan rate of 10 ^{o}C/min.
The morphology of the samples was recorded using Scanning Electron Microscopy (SEM) in a Hitachi S-2400 instrument, with an accelerating voltage set to 15 kV. All samples were gold coated before analysis.
X-ray photoelectron spectroscopy (XPS) analysis were performed on a XSAM800 X-ray spectrometer, operated in the fixed analyser transmission (FAT) mode, with a pass energy of 10 eV, a power of 120 W and using a non-monochromatic radiation (energy of 1486.6 eV). Spectra were collected with a step of 0.1 eV, using a Sun SPARC Station 4 with Vision software (Kratos). The curve fitting for component peaks was carried out with a non-linear least-squares algorithm using a commercial program – the XPSPEAK41. For the bond energy correction (E_L) for accumulated charge, was considered the bond energy of bonded carbon only for other carbons and for hydrogen of 285 eV. Sensitivity factors used were: C 1s - 0.25, O 1s 0.66, N 1s - 0.42 and Si 2p – 0.27.
Elementary analysis was achieved using a FlashEA 1112 Series CHNS Analyzer. The tests were performed with the combustion reactor temperature of 900 ^{o}C, the GC column temperature of 65 ^{o}C, the Helium flow rate of 130 ml/min, the oxygen flow rate of 250 ml/min. The oxygen injection time was 7 s, the sample injection time was 12 s and the analysis time was 720 s. The sample and standard height was 2-3 mg. The calibration

standard was BBOT [2,5-Bis (5-tert-butyl-benzoxaxol-2-yl) thiophene] 6.51% N, 72.53% C, 6.09% H e 7.44% S, with K factor as calibration method.

1.2.3. *In-situ* polymerization of 2-substituted oxazolines

1.2.3.1. Experimental apparatus

The *in-situ* polymerization was undertaken in a high-pressure apparatus schematically presented in Figure 1.8. The real apparatus is shown on Figure 1.9, Figure 1.10 and Figure 1.11.

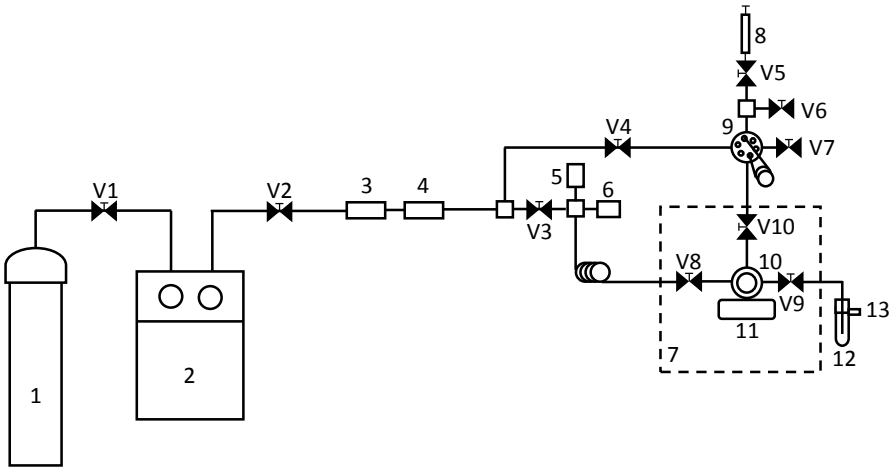


Figure 1.8 Schematic representation of the experimental apparatus. 1-CO_2 cylinder; 2-high pressure pump; 3-line filter; 4-check valve; 5-high pressure transducer; 6-rupture disk; 7-thermostatted bath; 8-syringe; 9-HPLC high pressure valve; 10-high-pressure cell, 11-stirrer; 12-shlenk; 13-vent; V1 to V10-high pressure valves (adapted from reference[29])

Figure 1.9 Real experimental apparatus, during the in-situ polymerization.

Figure 1.10 ***Real apparatus of a high-pressure cell.***

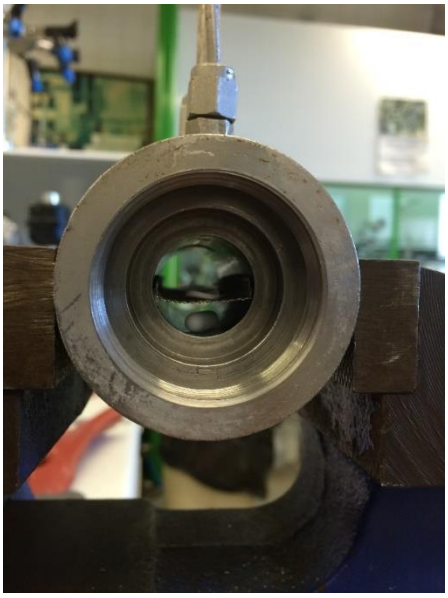


Figure 1.11 ***Real experimental apparatus, inside the high-pressure cell.***

1.2.3.2. Pre-treatment of the PE samples

The polyethylene-based vaginal applicator was cut into similar pieces (~25 mg) and the samples were washed with ethanol under stirring overnight.

1.2.3.3. Synthesis of *living oligomers*

In situ-polymerization reactions were carried out in a 33 ml high-pressure cell. Two different 2-substituted oxazoline oligomers were synthesized (MeOx and EtOx) and boron trifluoride etherate ($BF_3.Et_2O$) was used as initiator. The monomer/initiator ratios

used were [M]/[I]=15 (2-methyl-2-oxazoline) and [M]/[I]=12 (2-ethyl-2-oxazoline), according with previous studies.[29]

The PE samples (~25 mg), the 2-substituted oxazoline and the initiator were placed in a high-pressure cell with a magnetic stirring bar, with a physical division between the PE samples (see Figure 1.11) and the reagents, and then immersed in a thermostatized oil bath. The polymerizations were performed at 115 ºC. The desired pressure of 18 MPa was achieved using carbon dioxide pressurized in the high-pressure cell. After 20 h of reaction, the *living oligomer* was obtained and able to be terminated with different molecules.

1.2.3.4. End-capping with *N,N*-dimethyldodecylamine

The termination of *living oligomer* was performed with addition of a tenfold excess of amine in relation to the added amount of initiator,[48] using a HPLC high-pressure valve. The reaction was maintained at the temperature of polymerization under stirring during 24 h. After this, the temperature was lowered to 45ºC and the oligomer was washed using fresh CO_2 during 2 h, in order to remove unreacted reagents. The obtained samples were placed in ethanol overnight under stirring, in order to remove the oligomer adsorbed to the surface (see Figure 1.12).

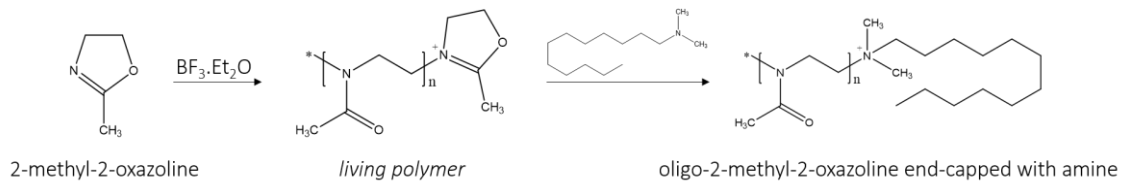


Figure 1.12 Synthesis of oligo(2-methyl-2-oxazoline) end-capped with N,N-dimethyldodecylamine using CROP polymerization in scCO₂ medium.

1.2.3.5. Impregnation of Lavender oil

PE samples (~25 mg) were impregnated with lavender oil in $scCO_2$ using a high-pressure cell. The impregnation was performed at 115 ºC and 18 MPa using an excess of oil. After 20 h of continuous stirring the high pressure vessel was rapidly depressurized.

The lavender oil used was extracted using $scCO_2$ in the aim of a doctoral thesis[1] at the host laboratory and was used in the development of these materials. Composition of lavender oil extracted can be found in Appendix 1.

[1] Work developed by Pedro Lisboa. Lavender gently offered by Delta S.A.

1.2.4. Evaluation of the antimicrobial activity

1.2.4.1. Microorganisms growth conditions

Escherichia coli DH5α (gram-negative bacteria), *Staphylococcus aureus* ATCC25923 and *Staphylococcus aureus* COL MRSA (both gram-positive bacteria) strains were grown at 37ºC in Mueller-Hinton broth medium (MHB, DIFCO, USA). *Candida albicans* PYCC 3436 and *Candida glabrata* PYCC2814 (both yeasts) strains were grown at 37ºC in Yeast Mannitol broth medium (YMB, composition in g/L: Potassium hydrogen phosphate 0.5; magnesium sulfate heptahydrate 0.2; sodium chloride 0.2; calcium chloride hexahydrate 0.2; mannitol 10; yeast extract 0.4). Cultures grown overnight at 37ºC were diluted in the same media to 10^5 CFU/mL to carry out the tests referred to below.

1.2.4.2. Disc diffusion

This test was performed using the non-impregnated oligomers recovered from the bottom of the reactor after the *in-situ* polymerizations. Cells were cultivated using a swab impregnated with a solution of each microorganism (10^5 cells) in standard growth medium, Mueller-Hinton Agar (MHA, DIFCO, USA).

Oligomers were dissolved in sterile water in a concentration of 100 mg/ml. Lavender oil was used without dissolution.

Paper discs (no. 231039, Becton and Dickinson, USA) were then impregnated with 10 µl of oligomer solution or lavender oil and placed on the surface of the agar plates containing growth medium. A negative control was also performed using a disc impregnated with sterile water. The plates were incubated for 24 h at 37ºC for bacterial strains and 48 h at 37ºC for yeast. Experiments were executed in duplicate and after incubation the inhibition zone was evaluated.

1.2.4.3. Microdilution

The tests were performed using four different microorganisms referred to above: *E. coli*, *S. aureus* COL, *C. albicans* and *C. glabrata*. One impregnated polyethylene sample (~25 mg) was placed in each well of a 24-well tissue culture plate, containing 1 ml of medium and 5 µl of the microorganism suspension (10^5 cells), and incubated at 37 °C with shaking for 24 h for bacterial strains and 48 h for yeast. The samples were diluted (10^{-1} to 10^{-8}) and 20 µl of selected diluted cultures were plated on MHA for bacterial strains,

and Yeast Mannitol Agar (YMA, composition in g/L: Potassium hydrogen phosphate 0.5; magnesium sulfate heptahydrate 0.2; sodium chloride 0.2; calcium chloride hexahydrate 0.2; mannitol 10; yeast extract 0.4; agar 10) for yeasts, and incubated at 37°C for 24 h. After this, the number of colonies was determined (colony forming units – CFU/ml) and the results were normalized for reduction of the viable cells in log stages (see Figure 1.13). Experiments were performed in duplicated in two independent assays.

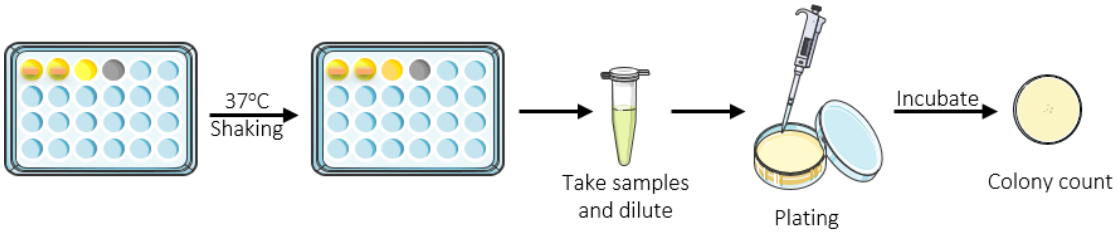


Figure 1.13 Schematic representation of the methodology used to evaluate the antimicrobial activity of samples.

The long term stability of the samples was evaluated using an Accelerated Aging procedure (ASTM F1980). Data obtained from the study is based in the conditions that simulate the effects of aging on the materials.

Accelerated Aging calculation is based on Arrhenius' equation which simply states that a 10 °C increase in temperature doubles the rate of chemical reaction. Four variables were used in calculating the accelerated aging test duration: test temperature (Te), ambient temperature (Ta), Reaction rate factor (Q_{10}=2 for medical devices) and real-time equivalent (DRT).

$$AAR(Accelerated\ Agging\ Rate) = Q_{10}^{((Te-Ta)/10)} \quad \text{eq. 1}$$

$$AATD(Accelerated\ Agging\ Time\ Duration) = \frac{Desired\ Real\ Time}{AAR} \quad \text{eq. 2}$$

Using equations 1 and 2, it was possible to determine the conditions for the accelerated aging test. In this case, the samples were placed at 60 °C during 52 days. After this time, the bacteria viability was tested using the same procedure as described before.

1.2.4.4. Bactericidal vs bacteriostatic

Using the mediums from microdiluition test where bacterial growth was not verified, 10 µl were diluted in 1 ml of new medium and incubated at 37 ºC during 24 h. After this time, the presence of growing was evaluated.

1.2.5. Evaluation of the anti-biofouling activity

The samples used in the microdilution tests were washed with PBS and fixed with glutaraldehyde (2.5%) during 10 minutes. After that, the samples were washed with water, dehydrated with ethanol 70%, 80% and 90% sequentially during 10 minutes and stored in ethanol 100%. SEM was used to assess the bacterial adhesion and proliferation during the 24 h of the microdilution assay for bacteria or 48h for yeasts.

1.2.6. Biocompatibility tests

1.2.6.1. Polyethylene impregnated with 2-substituted oxazolines

In vitro cytotoxicity assays were performed with cell line HCT-116 (colorectal carcinoma cell line) and human fibroblasts through the use the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA), a colorimetric method for determining the number of viable cells.

To the maintenance and splitting of the cell line, and at 80 % confluence, cells were harvested and centrifuged during 5 minutes. The supernatant was discarded, and the cell pellet resuspended in 2 ml of medium. For growth inhibition assays, $0.75x10^5$ cells/mL were plated into flat bottomed 96-well plates (Costar, Corning, NY) and incubated at 37 ºC, 99% humidity and 5% CO_2 (v/v). Cell density was evaluated as the total number of viable cells within the grids on the hemacytometer (Hirschmann, Eberstadt, Germany) using trypan blue exclusion method. For this procedure 350 µl of medium was pipetted to a 2 mL eppendorf together with 50 µl of the 2ml cell suspension followed by 100 µl of 0.4 % (v/v) trypan blue solution (Sigma). The hemacytometer was loaded and examined immediately under the microscope at low resolution, and cell viability was determined through the following equation:

$$\frac{Nº\ of\ cells}{ml} = \frac{\sum cells\ per\ quadrant}{4} * 10^4\ (Chamber\ Volume * Dilution\ factor) \qquad \text{eq. 3}$$

After 24 h, MeOx and EtOx solutions (0.45 mg/mL), and PE, PE_MeOx and PE_EtOx material samples were added (after removal of depleted medium), and the cells were incubated for 1 h and 24 h [37 ºC, 99% humidity and 5% CO_2 (v/v)]. Subsequently a reaction mix of medium, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), and PMS (phenazine methosulfate) (Kit Promega) in a ratio of 100:19:1 was added to each well and further incubated for 45 min. During this period, MTS is bioreduced into formazan, by dehydrogenase enzymes present in metabolically active cells, which in turn is susceptible of being measured at 490 nm absorbance by Tecan Infinite F200 Microplate Reader (Tecan, Männedorf, Switzerland), directly from the 96-well assay plate, so that the quantity of formazan product measured is directly proportional to the number of living cells in culture. The cell viability results for each concentration were normalized relatively to the control samples and obtained accordingly to the following formula:

$$\frac{Sample\ Absorvance\ (490\ nm)}{Control\ Absorvance\ (490\ nm)} * 100 = Cell\ Viability\ (\%) \qquad \text{eq. 4}$$

1.2.6.2. Polyethylene impregnated with lavender oil

1.2.6.2.1. Proliferation of human fibroblast cells in the presence of samples

To evaluate cell behavior in the presence of the samples, human fibroblasts cells ($4x10^4$ cells/well) were seeded with materials in a 96-well plate and cultured with DMEM-F12 supplemented with fetal bovine serum (FBS), for 48 h, at 37°C under a 5% CO_2 humidified atmosphere. Wells containing cells in the culture medium without materials were used as negative control. EtOH 96% was also added to some wells and they were used as a positive control. Previously to cell seeding, materials were sterilized using UV irradiation during 30 minutes. Cell growth was monitored using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera.[49,50,51]

1.2.6.2.2. Characterization of the cytotoxic profile of the samples

Human fibroblast cells were seeded, in contact with sterilized materials, at a density of $4x10^4$ cells/well and cultured with DMEM-F12. Subsequently, the mitochondrial redox activity of viable cells was assessed through a resazurin assay (n=5). At 24 and 48 h, cells were incubated with 100 µL DMEM-F12 and 10 µL of resazurin 0.1% (w/v) in 5%

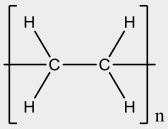
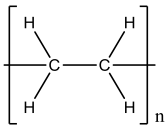
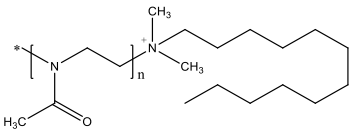
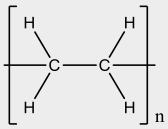
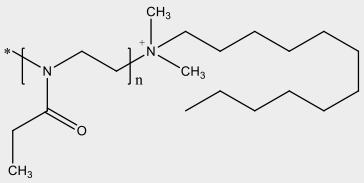
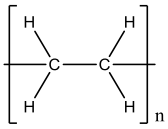
CO_2 humidified incubator, for 24 h, at 37 ºC. Fluorescence of metabolized resazurin was measured using a Gemini EM spectrophotometer at an excitation/emission wavelength of λ=545/590nm, respectively.[52] Wells containing cells in the culture medium without materials were used as negative control (K-). Ethanol (96%) was added to wells that were used as a positive control (K+).[49,53]

1.3. Results and discussion

1.3.1. Synthesized materials

Figure 1.1 summarizes all synthesized and used materials and their respective compositions.

Table 1.1 ***Reference and composition of materials.***

Material abbreviation	Polymer matrix	Antimicrobial compound
PE	Polyethylene 	
PE_MeOx	Polyethylene 	Oligo(2-methyl-2-oxazoline) end-capped with N,N-dimethyldodecylamine 
PE_EtOx	Polyethylene 	Oligo(2-ethyl-2-oxazoline) end-capped with N,N-dimethyldodecylamine 
PE_OA	Polyethylene 	Lavender oil (composition in Appendix 1)

1.3.2. Materials characterization

1.3.2.1. Gravimetric determination of loading

All polymerizations were carried out at 115 ºC and 18 MPa during 20 h and more 24 h for oligomer end-capping.[30,54] High density polyethylene is extremely difficult to process or blend, due to its high molecular weight and high degree of chain entanglement.

The gravimetric data were obtained by the following equation:

$$Mass\ gain\ (\%) = \frac{W_t - W_0}{W_0} \times 100 \qquad \text{eq. 4}$$

where W_0 is the initial weight of the PE sample and W_t is the weight of the modified PE sample after washing and drying. The reported mass gain was the mean value of six different samples.

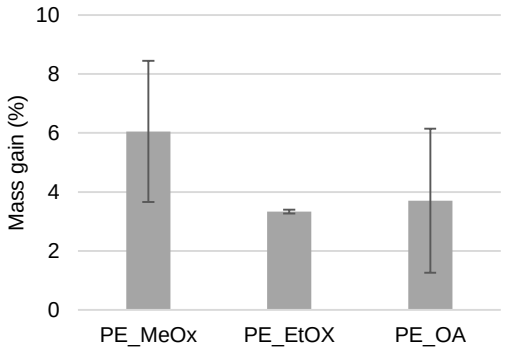


Figure 1.14 ***Variation of the mass gain (%) in modified PE samples for different antimicrobial compounds used.***

Observing the results for the modified PE samples with oligo(2-oxazoline)s (Figure 1.14), there is a decrease in the mass gain of PE samples with the increase in the size of the side chain of the oligo(2-oxazoline). The amount of monomer used was calculated in order to achieve the saturation of medium with monomer, ensuring the maximum loading into the PE matrix. The PE modified with oligo(2-methyl-2-oxazoline), comprising a methyl side chain, presents a higher mass gain comparing with PE modified with oligo(2-ethyl-2-oxazoline), which contains an ethyl side chain. This suggests that the rate of diffusion through the PE matrix plays a role in the loading of the monomer, once all the reaction conditions were the same, the free volume of PE is identical and hence the larger monomers diffuse through the matrix slower than smaller ones, leading to a small amount of oligomer polymerized inside the PE matrix.

In the case of PE impregnated with lavender oil, there is no previous work describing the supercritical-assisted impregnation in literature, so it is not possible to compare this result with previous data. Although, PE impregnated with lavender oil presents similar values of mass gain to the modified PE samples with oligo(2-oxazoline)s showing that probably that is the maximum amount possible to impregnate in this polyethylene matrix.

1.3.2.2. Morphological characterization

SEM micrographs were obtained for unmodified PE, PE modified with two 2-oxazoline-based oligomers and PE impregnated with lavender oil (see Figure 1.15).

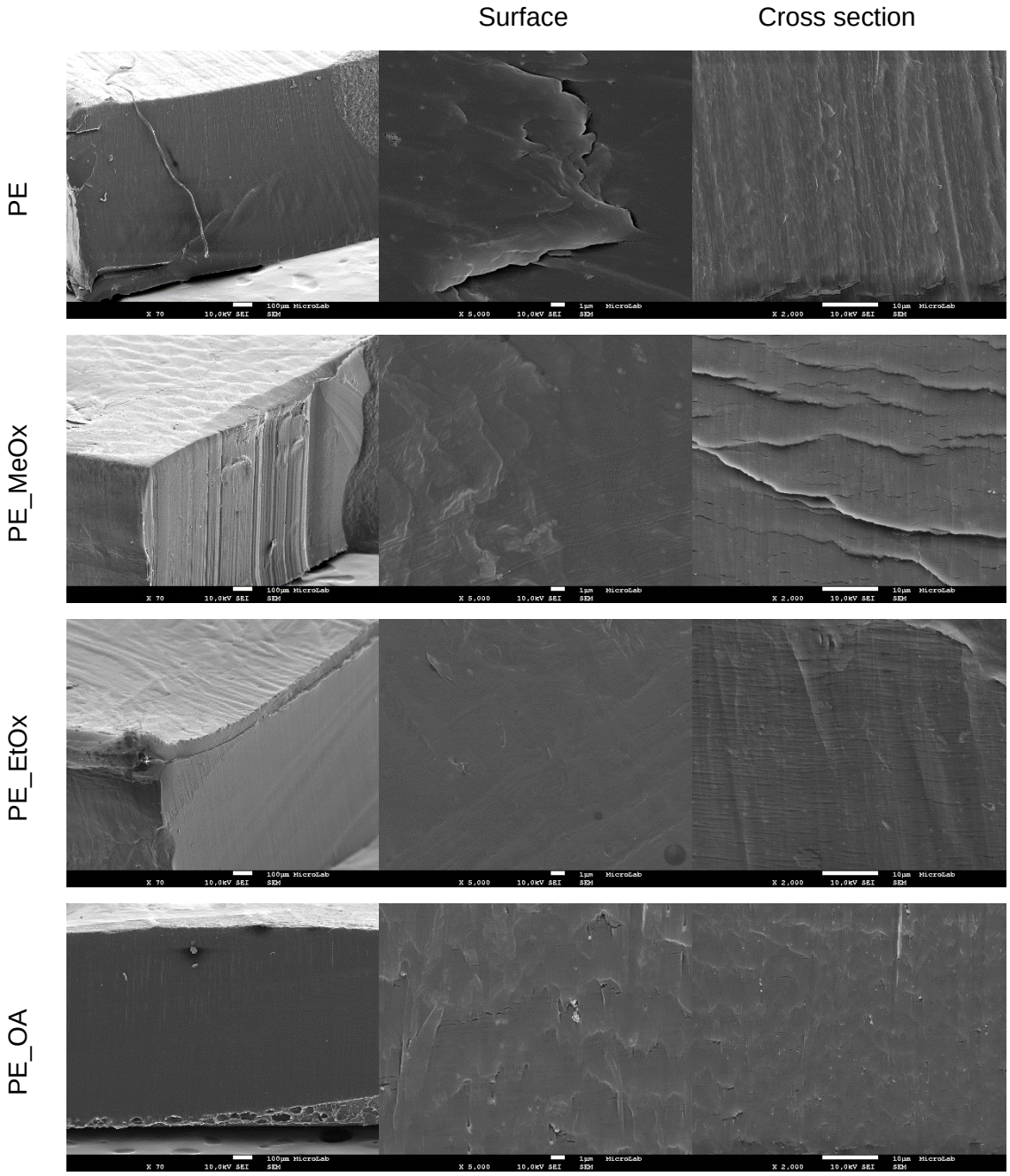


Figure 1.15 SEM micrographs of surface and cross section of the unmodified PE and PE modified with antimicrobial compounds: PE_MeOx, PE_EtOx and PE_OA.

The SEM images show that all materials present the same morphology, without changes after the supercritical-assisted modification. These results confirm that processing

material in high pressure and temperature conditions do not cause morphological modifications in the structure. Moreover, the presence of oligomers or compounds are not visible at the PE matrix surface, suggesting that the antimicrobial compounds are inside the PE matrix, which was swelled during the $scCO_2$ process and back to original form after depressurization, confining the new compound inside and avoiding its leaching.[46]

1.3.2.3. Determination of materials composition

In order to confirm the success of oligomers polymerization, NMR analysis was made using the non-impregnated oligomers recovered from the bottom of the reactor after the *in-situ* polymerizations (see Appendix 2 and Appendix 3).[30]
The effectiveness of the supercritical *in-situ* polymerization and impregnation procedures was evaluated by XPS and elementary analysis.

Table 1.2 ***Percentage of different elements in the surface of each sample. Data obtained by XPS.***

Sample	Nitrogen (%)	Carbon (%)	Oxygen (%)	Silicon (%)
PE	2.1	80.0	16.5	1.4
PE_MeOx	3.7	81.9	11.7	2.7
PE_EtOx	0.3	83.8	14.1	1.7
PE_OA	0.9	84.4	13.0	1.6

XPS is a technique able to identify and quantify the elemental composition at the surface region with an analysis depth of the order of 3-10 nm. Observing the results (see Table 1.2), no significant changes were observed comparing the untreated PE with modified PE materials. This technique only analyses the surface, and most of this differences could be due to the presence of some impurities at the samples surface, which are not significant when considering the overall sample.

Table 1.3 ***Percentage of different elements in each sample, evaluated by elemental analysis.***

Sample	Nitrogen (%)	Carbon (%)	Hydrogen (%)	Sulphur (%)
PE	0.03	83.12	11.86	0
PE_MeOx	0.12	82.92	13.58	0
PE_EtOx	0.07	83.09	12.80	0
PE_OA	0.06	82.54	13.43	0

Complementing the XPS analysis, elemental analysis based on sample combustion dynamics was performed. Analyzing the Table 1.3, we could notice an increase in the amount of nitrogen and hydrogen, confirming the introduction of a new compound containing these elements in its composition. Also, it is possible to see a decrease in the percentage of carbon (the element present in more quantity in PE), which is an expected result, since the addition of new compounds to the PE matrix reduces the proportion of this element in the overall matrix.

1.3.2.4. Thermal properties

DSC thermograms of unmodified PE, of 2-oxazoline-based oligomers synthesized outside the PE matrix and of modified PE samples are shown in Figure 1.16.

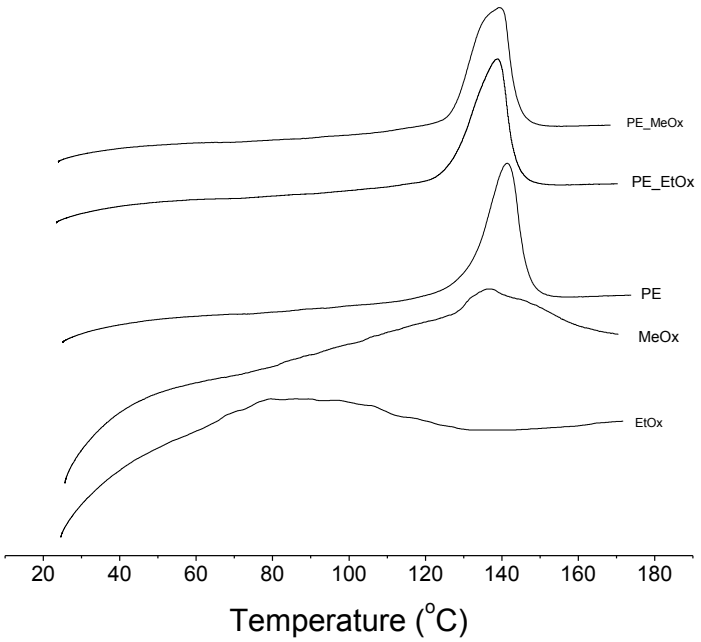


Figure 1.16 DSC thermograms of unmodified PE, of oligomers synthesized outside the PE matrix and of modified PE samples.

The DSC of unmodified PE displays the expected single melting peak at 139 ºC. By analysing the thermograms, we can conclude that the presence of oligo(2-oxazoline)s oligomers in the PE matrix shift melting temperature (T_m) to values more close of oligomer T_m and also this last one disappear. It can be seen that in each thermogram only one T_m is present, losing the peak corresponding to the oligomer, which suggests

an interaction between the oligomer and PE matrix, and consequently a successfully impregnation. These results also suggest that the treatment with $scCO_2$ do not change the degree of crystallinity compared with original PE.

1.3.3. Evaluation of the antimicrobial activity

1.3.3.1. Disc diffusion

In order to evaluate the susceptibility of bacteria and yeasts to the developed antimicrobial compounds, solutions of 100 mg/mL in water of each oligomer were placed in paper discs and tested for different microorganisms. Also, lavender oil extracted using $scCO_2$ was tested against the same microorganisms, without any dilution. This method allows a quick determination of the antimicrobial activity of compounds.

Table 1.4 Antimicrobial activity evaluated by disk diffusion for different synthesized oligomers and lavender oil, against different gram positive and negative bacteria and yeasts.

	Diameter of growth inhibition zone (mm)		
Microorganism	**MeOx**	**EtOx**	**Lavender oil**
E. coli DH5α	15	17	12
S. aureus COL	20	23	23
S. aureus ATCC 25923	29	31	22
C. albicans PYCC 3436	29	36	10
C. glabrata PYCC 2814	15	23	0

From the Table 1.4 it is possible to say that the synthesized oligomers, oligo(2-methyl-2-oxazoline) and oligo(2-ehtyl-2-oxazoline), have a significant antimicrobial activity against all the 5 microorganisms tested. Oligo(2-ethyl-2-oxazoline presents for all the microorganisms the highest growth inhibition zone. This results are in agreement with previous reports using similar oligomers.[30] This kind of test only gives a qualitative result, since the limitations of the technique should be considered, such as the diffusion of the solution from the disk due to the viscosity or possible interactions between the sample and the disk.

In the case of lavender oil, antimicrobial activity was observed against all microorganisms, with exception of *C. glabrata*. The chemical composition of lavender oil is reported in Appendix 1 and as expected, linalool and linalyl acetate were the main

components. Linalool has been reported as the component responsible for the yeastscidal activity and the activity of lavender oil against microorganisms could be depend on an additive effect of its major components; however, the contribution of minor constituents to the antimicrobial activity cannot be disconsidered.[55] A previous study already reported the antimicrobial and antifungal activity of lavender oil.[56] The result related with *C. glabrata* is unexpected, once the activity against yeasts is already reported. However there is no previous study reporting the antimicrobial activity of the lavender oil against this microorganism. This occurrence could be due to the previously mentioned limitations of the technique and a negative result can not mean the absence of activity and this result should be confirmed with tests performed in liquid medium.

It is important to note that the tested compounds present antimicrobial activity against a multi-resistant *S. aureus*, which is a very promising result, since there are not many compounds effective against this microorganism.[57]

Unfortunately, there is no zone diameter interpretative standards for the tested compounds, so it is not possible to evaluate if the microorganisms are susceptible, intermediate or resistant.

1.3.3.2. Microdilution

To evaluate the ability of the materials to kill bacteria and yeasts upon contact, cells viability after direct exposure to PE modified with antimicrobial compounds was assessed using *E. coli*, *S. aureus*, *C. albicans* and *C. glabrata*.

The materials were incubated in medium containing cells and, after incubation, samples medium were diluted, plated and the number of colony-forming units (CFU) was counted. Incubation of bacteria and yeasts cells, with stirring, in the presence of the unmodified PE did not influence the normal growth of the *E. coli*, *S. aureus* and *C. glabrata* cells. These results are the expected, since PE in its native form does not have antimicrobial activity (Figure 1.17).[58] However, unmodified PE killed >99.9% of the *C. albicans* cells (3 log stages reduction=a reduction of 99.9%). This effect was not predictable, so other tests are required to explain this result, such as the use of another strain of *C. albicans* to clarify if this antimicrobial activity is only for this strain in particular or species-specific. The addition of 2-oxazoline-based antimicrobial oligomers to the PE matrix improved drastically the material antimicrobial performance. After 18 h for bacteria and 24 h for yeasts, the materials reduce the cells viability over 99.9%. These results are in accordance with previous data reported for materials comprising similar compounds [59].

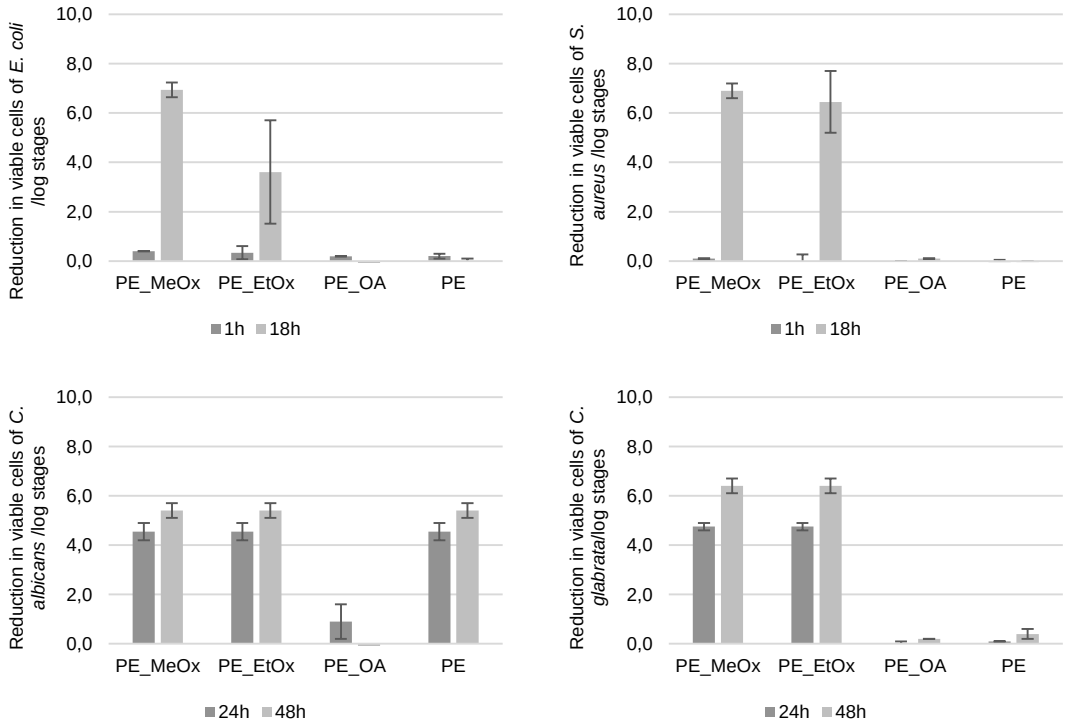


Figure 1.17 ***Inhibition of E. coli, S. aureus, C. albicans and C. glabrata replication (left to right, top to bottom) by direct exposure to different materials with antimicrobial activity. The results are presented as the reduction in number of viable cells in a logarithmic scale.***

The presence of positive charge is an important requirement for the antimicrobial activity of the samples due to the mechanism of action, since the phospholipid content of membranes is predominantly anionic. As previous suggested, the cationic extremity of the oligomer should initiate electrostatic interactions with the negatively charged membrane of microorganisms and consequently leading to its disruption.[31]

The addition of lavender oil to the PE did not improve the antimicrobial properties of the material. Since *E. coli, S. aureus* and *C. albicans* showed susceptibility to this compound in the disc diffusion technique, it is possible that something during the impregnation affected its activity. It is possible that the amount of oil that diffused to the matrix was lower than the necessary amount to kill these microorganisms, or some compounds of lavender oil might have partitioned differently to the $scCO_2$ phase leading to a non-efficient impregnation of the more active compounds, since the $scCO_2$-assisted extraction was made at different conditions.[55]

The long term stability of the samples was evaluated using the same method as previously, after 52 days at 60 ºC. After this treatment, few changes in antimicrobial activity of the materials were noticed (see Figure 1.18).

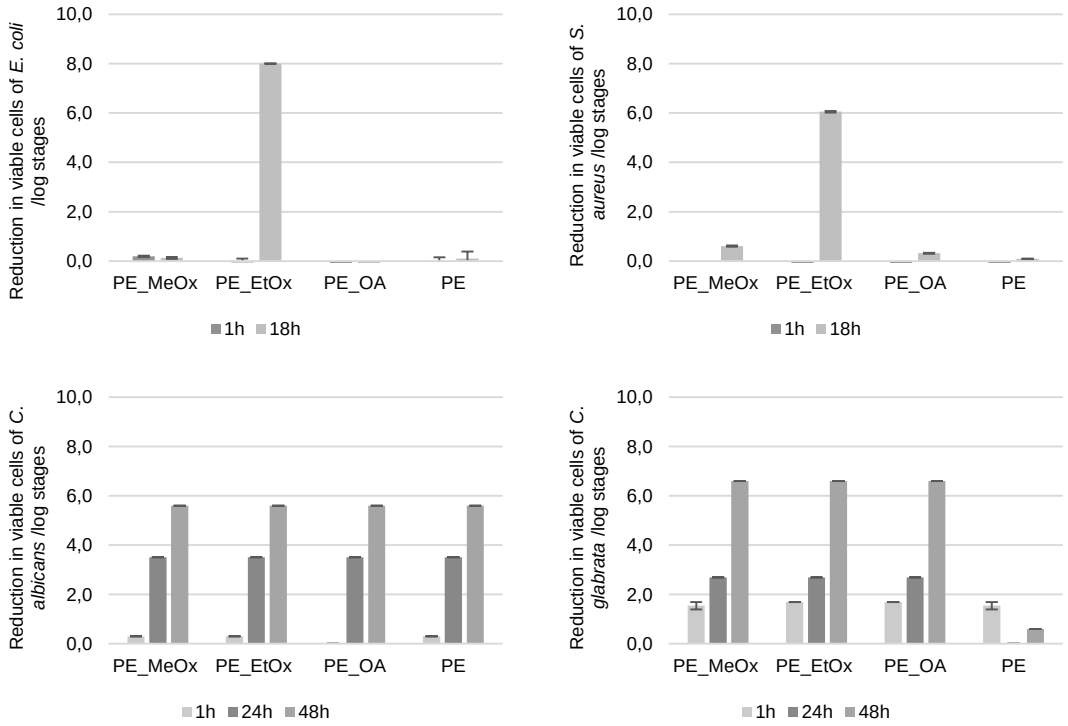


Figure 1.18 ***Evaluation of the long term stability of samples, by assessment of the antimicrobial activity using the same method as before, after 52 days at 60 ºC. Killing of E. coli, S. aureus, C. albicans and C. glabrata (left to right, top to bottom) by direct exposure to different materials with antimicrobial activity.***

A loss in activity of PE_MeOx against *E. coli* and *S. aureus* was verified, suggesting that this compound suffered some changes during this time at these conditions that unable its capacity to kill these microorganisms. It is possible that the loss in activity may be due to degradation of oligomer or to a lower binding affinity to the bacterial cell membrane or to limited diffusion through the bacterial cell wall.

On the other hand, the PE_OA material acquired antimicrobial activity against *C. albicans* and *C. glabrata*. This could be explained by decomposition of some compounds of the lavender oil, making it toxic for cells and killing them.

The PE_EtOx did not show any variation in its antimicrobial activity, demonstrating stability at these conditions.

1.3.3.3. Evaluation of the anti-biofouling activity

Biofouling is a multistage process that starts with the formation of a biofilm in which microorganisms adhere to the surface. A compound that does not kill microorganisms, can prevent the formation of a biofilm by, for instance, changing only the materials hydrophilicity. In this work, we changed the materials hidrophilicity at the same time that some antimicrobial characteristics to the material were introduced. Even if the modified

material is not able to release the antimicrobial compound to the medium to avoid bacterial contamination, the material can be able to avoid bacterial growth and proliferation at the surface. In order to evaluate if PE modified with antimicrobial compounds presents any anti-biofouling activity, the samples used in the microdilution tests were placed in glutaraldehyde to fix the microorganisms, dried with ethanol and the surfaces were analysed by SEM.

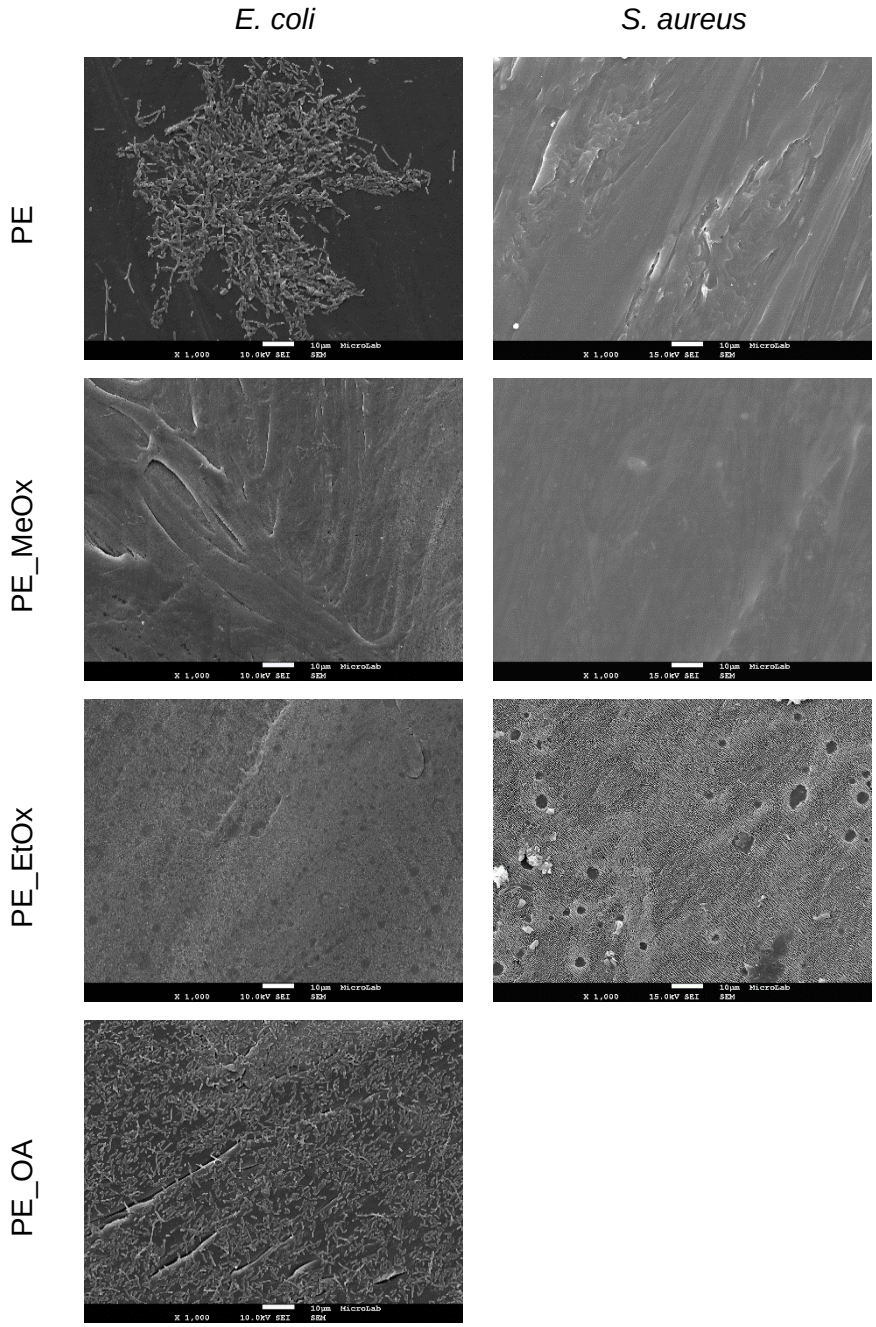


Figure 1.19 SEM micrographs of surface of the PE, PE_MeOx, PE_EtOx and PE_OA after contact with E. coli and S. aureus.

The absence of biofilm formation for the material modified with oligo(2-oxazoline)s was already expected, once these compounds have shown a high capacity to kill these microorganisms in the evaluation of antimicrobial activity (Figure 1.19 and Figure 1.20)

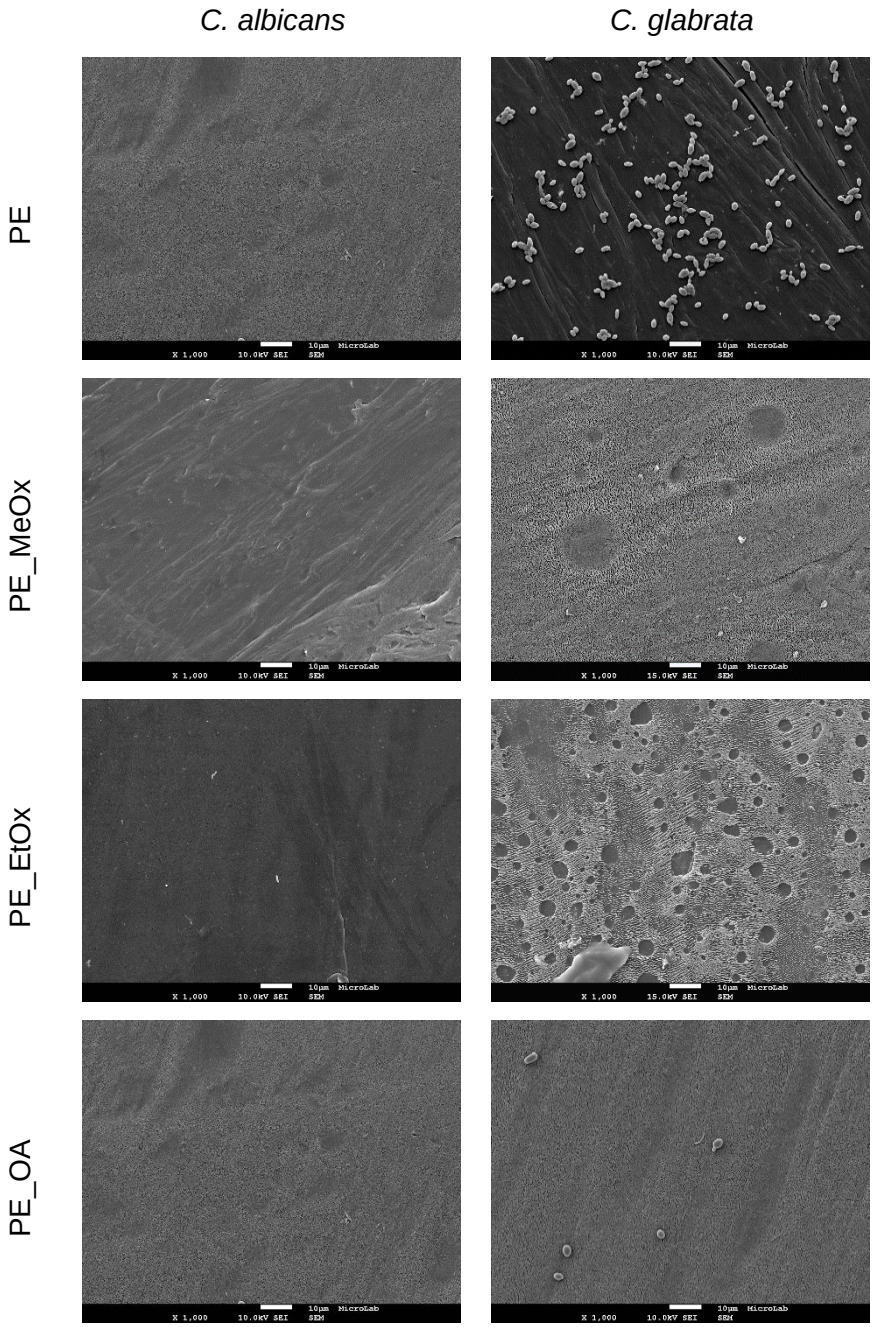


Figure 1.20 SEM micrographs of surface of the PE, PE_MeOx, PE_EtOx and PE_OA after contact with C. albicans and C. glabrata.

For unmodified PE the prevention of the biofilm formation was expected only for *C. albicans*, since that PE have shown to be active against this microorganism. However, PE also prevented the formation of biofilm for *S. aureus*. None of these results for anti-biofouling activity of unmodified polyethylene is in accordance with the literature, since the previous studies reported that surface of polyethylene is easily colonized by microorganisms.[60]

In the case of polyethylene impregnated with lavender oil, despite did not present antimicrobial activity for any microorganism, it was effective in inhibiting biofilm formation for both yeasts, *C. albicans* and *C. glabrata*. These results are in accordance with previous reports, which have refer that lavender oil is effective against yeasts.[55] The quantity of lavender oil that diffused to the liquid medium could be not enough to kill bacteria but the impregnation revealed to be enough at least to inhibit yeasts adhesion at the surface.

The anti-biofouling activity of this material for *S. aureus* could not be assessed, since this sample was degraded during SEM analysis.

1.3.3.4. Bactericidal or bacteriostatic

In order to evaluate if the antimicrobial compounds, that have shown to be effective, really kill the microorganisms by destroying the membrane (bactericidal) or just prevent the growth of bacteria inhibiting its multiplication (bacteriostatic), a test was performed. In the microdilution test, no microorganisms growing were observed for polyethylene modified with oligo(2-oxazoline)s. In this test, medium samples of each previous well were taken and placed in contact with new medium, and the presence of microorganisms growing after 24 h was evaluated.

Table 1.5 ***Evaluation of the presence of growing using samples of medium obtained in microdilution test in new medium.***

	PE	PE_MeOx	PE_EtOx
E. coli	-	No	No
S. aureus	-	No	No
C. albicans	Yes	No	No
C. glabrata	-	No	No

Observing the growing results (Table 1.5), it is possible to conclude that the antimicrobial compounds are bactericidal, once there is absence of microorganisms growing when samples of medium with no growing in the microdilution test were placed in new medium

and stored at 37ºC during 24 h. These results are in accordance with literature about the possible mechanism of action of similar polymers, suggesting that the main target of these agents is the microorganisms membrane, causing disruption by the linear end-capper group.[30,61,62] In the case of PE active against *C. albicans*, the activity is bacteriostatic, just inhibiting the multiplication in the presence of the compound. As previously mentioned, this result is not in accordance with previous reports about antimicrobial activity of pure polyethylene.[58]

1.3.4. Biocompatibility tests

In order to evaluate the applicability of these new materials for biomedical applications, the cytotoxicity was studied. Due to the need of specialized laboratories to do these tests, different materials were tested in different laboratories and different methodologies were used.

1.3.4.1. Polyethylene impregnated with lavender oil

Fibroblast cells were seeded at the same initial density in the 96-well plates, with and without sample materials to assess the cytotoxicity. After 48 h, cell adhesion and proliferation was visualized using an inverted light microscope (Figure 1.21). Cells grew in the vicinity of the both materials, PE_OA and PE, and in the negative control. In the positive control, no cell adhesion or proliferation was observed and a typical spherical shape of dead cells could be observed.

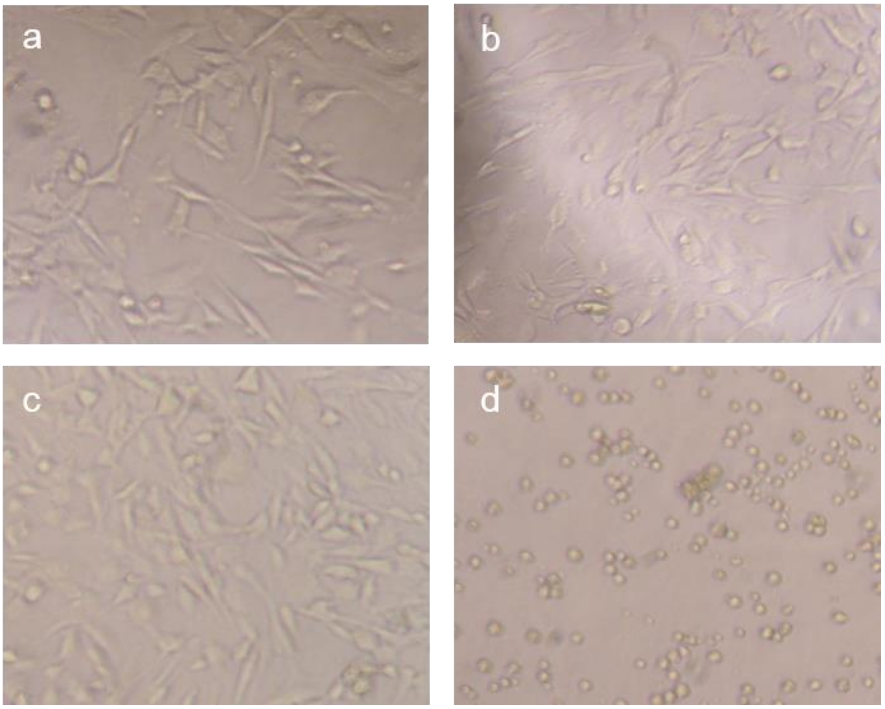


Figure 1.21 ***Photomicrographs of fibroblast cells after being in contact with PE_OA material (a) and untreated PE (b). Negative control (c), without addition of any component, and positive control (d), with addition of ethanol, is also presented.***

To further evaluate the biocompatibility of the materials, a resazurin assay was also performed (Figure 1.22). This test did not show a significant difference between cells exposed to materials and the negative control after 48 h of incubation, suggesting that the materials did not affect cell viability and do not have any cytotoxic effect.

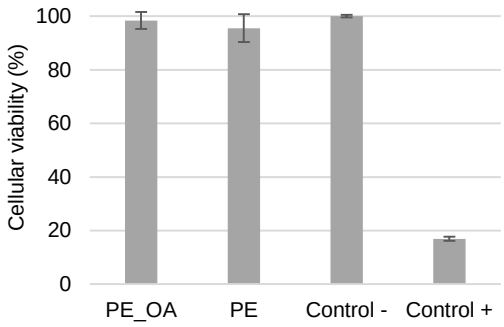


Figure 1.22 ***Cellular activity measured by the resazurin assay. Fibroblast cells in the presence of PE_OA material and untreated PE, negative control without addition of any component and positive control with addition of ethanol.***

1.3.4.2. Polyethylene impregnated with 2-substituted oxazolines

The cytotoxicity of PE_MeOx and PE_EtOx materials, untreated PE and MeOx and EtOx was characterized through *in vitro* studies using fibroblast cells and human colon

carcinoma HCT-116 cells after 1 h and 24 h of incubation. The analysis of the results was performed qualitatively and quantitatively.

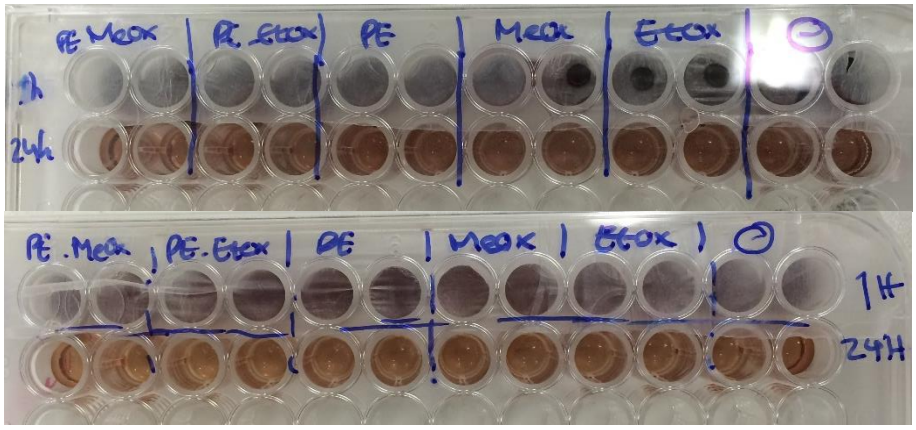


Figure 1.23 Visual observation of the microplates 45 min after addition of MTS for Fibroblast (above) and HTC-116 (bellow) after 1 h and 24 h of incubation, in the presence of PE_MeOx and PE_EtOx materials, untreated PE and MeOx and EtOx solutions and the negative control, respectively.

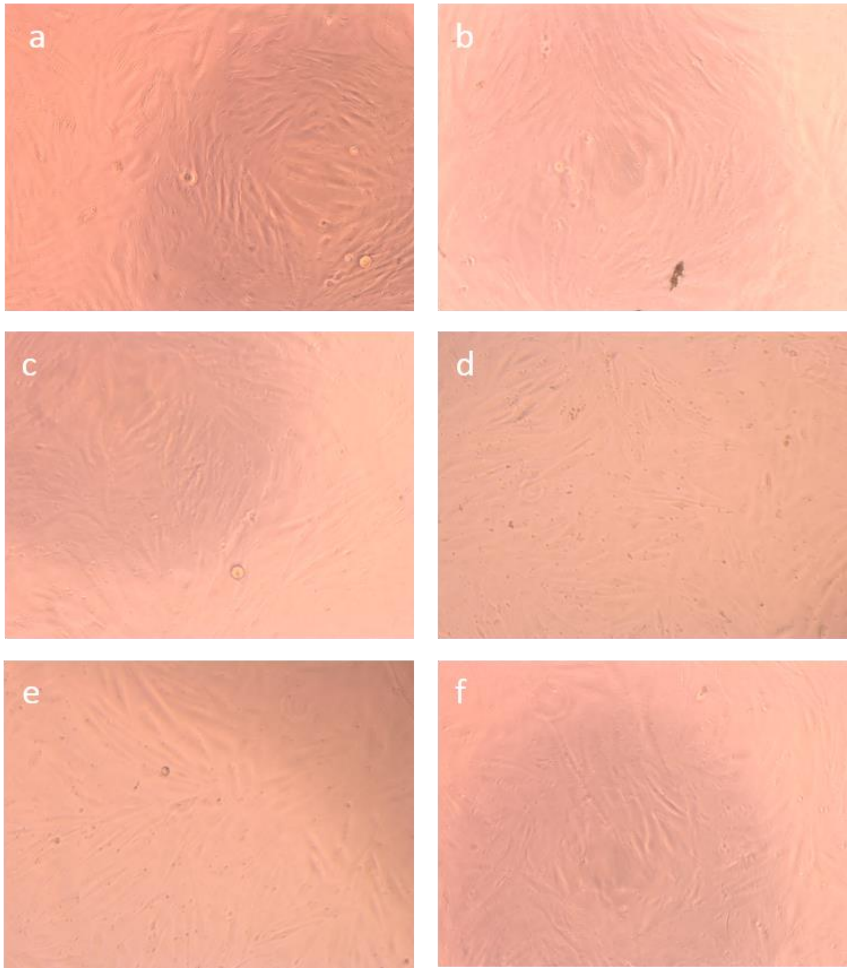


Figure 1.24 Photomicrographs of fibroblast cells after being seeded in contact with PE_MeOx (a), PE_EtOx (b), untreated PE (c), MeOx (d) and EtOx (e) during 24 h. Negative control (f), without addition of any component, is also presented.

By visual observation of the microplates (Figure 1.23) and photomicrographs of fibroblast cells (Figure 1.24) showing the cells shape, it is possible to infer about the biocompatibility of the tested materials and oligomers. The presence of reference color of the compound formed after reduction of the MTS reagent by the viable cells and the cells elongated shape give us a qualitative analysis suggesting any presence of cytotoxicity.

The quantitative analysis was made, by measuring the absorbance at 490 nm and the cell viability were normalized relatively to the negative control samples.

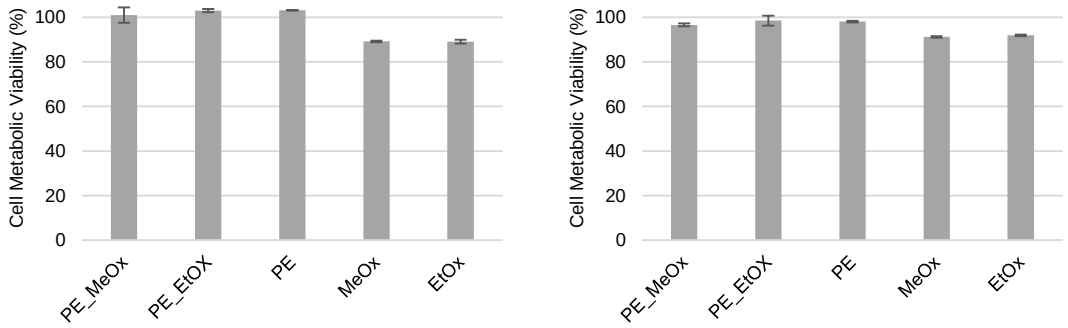


Figure 1.25 ***Cellular activity measured by the MTS assay. Fibroblast (left) and HTC-116 (right) after 1 h of incubation in the presence of PE_MeOx and PE_EtOx materials, untreated PE and MeOx and EtOx solutions.***

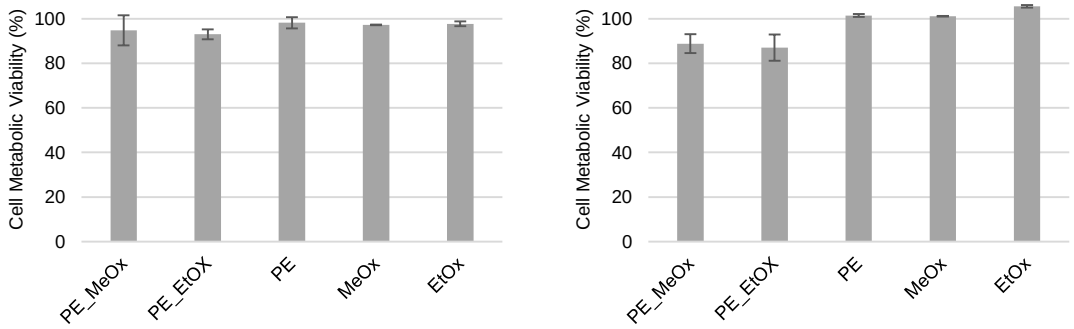


Figure 1.26 ***Cellular activity measured by the MTS assay. Fibroblast (left) and HTC-116 (right) after 24 h of incubation in the presence of PE_MeOx and PE_EtOx materials, untreated PE and MeOx and EtOx solutions.***

The results of the cytotoxicity evaluations after 1 h and 24 h of incubation (Figure 1.25) indicated that tested materials and oligomers displayed no cytotoxic behavior. These data of MeOx and EtOx oligomers are in accordance with the literature.[30] Also the results for PE are in agreement with previous studies reported.[63] The blend of compounds did

not affect their biocompatible behavior, showing a satisfactory biocompatibility of the developed materials.

1.4. Conclusion

In order to develop self-disinfecting polyethylene-based vaginal applicators, a modification with oligo(2-oxazoline)s and lavender oil was investigated to confer antimicrobial properties to the material. The *in-situ* polymerization of 2-substituted oxazolines via CROP using a $scCO_2$-assisted process and the end-capping with a quaternary amine led to a presence of antimicrobial oligomers inside the polyethylene matrix. Furthermore, polyethylene matrix was impregnated with lavender oil in order to confer the oil properties to the polymer. The presence of compounds in the matrix after washing was confirmed using XPS, elemental analysis and DSC. The ability to kill bacteria and yeasts upon contact was verified against *E. coli*, *S. aureus*, *C. albicans* and *C. glabrata*. Probably because of electrostatic interactions with the membrane of microorganisms and its disruption; polyethylene modified with oligo(2-oxazoline)s present a biocidal behavior, reducing the cell viability over 99.9%. The ethyl-oxazoline-based material remains biologically active against all microorganisms for at least 52 days.

On the other hand, polyethylene modified with lavender oil did not show effectiveness against the tested microorganisms. The reason for this extremely low activity could be the non-efficient impregnation of more active compounds of lavender oil, disabling the antimicrobial property of the material.

Moreover, direct contact cytotoxicity tests revealed that the polyethylene modified materials are highly biocompatible, do not showing any cytotoxic response.

The prepared polyethylene modified with oligo(2-oxazoline)s materials were more efficient than the polyethylene modified with lavender oil.

The oligo(2-oxazoline)s-modified polyethylene represents a promising material for biomedical applications due to the antimicrobial activity and biocompatibility. The use of such materials could minimize the impact of poor cleaning and disinfecting practices during usage.

For the industrial applications point of view of this antimicrobial compounds in the vaginal applicators, the direct incorporation of the active polymer during the device manufacturing process could be tested, in order to reduce the steps and the associated costs.

Chapter 2: Surface modification to reduce powder retention in a Dry Powder Inhaler

2.1. Introduction

This section describes an approach for inhaler's surface modification in order to reduce the powder retention and improve the lung delivery.

2.1.1. Inhalers

The respiratory tract is an attractive delivery route, with global drug delivery market size about 30%. The major advantages of pulmonary drug delivery include avoiding the severe conditions of the gastro intestinal, non-invasive path of administration, fast drug absorption and action, and local treatment of specific diseases.[64]

Novel inhaled therapeutics comprising vaccines, antibiotics and anti-hypertensives, have conducted to innovations in designing suitable delivery systems. These developing design technologies are in exigent demand to ensure high aerosolisation performance, consistent efficacy and satisfactory patient adherence.

The pressurised metered dose inhalers (pMDI) has been preferred by patients and healthcare professionals for the treatment of asthma and COPD since its introduction in 1956.[65] The active ingredients in pMDI inhalers are suspended or dissolved in a propellant, a mixture of propellants, or a mixture of solvents and they are delivered via a compact pressurized aerosol distributor. MDIs require the user to coordinate pressing down the canister and inhaling the drug. Another disadvantage of using pMDIs is the propellant, normally chlorofluorocarbon (CFC), which has been shown to deplete the ozone layer. Hydrofluoroalkane (HFA) is now used and can cause the inhaler to be more expensive.[66]

Nebulisers are useful for treatment of respiratory diseases as asthma, COPD and cystic fibrosis (CF).[67] They generate an inhalable drug aerosol from a solution or suspension. The most used nebuliser type is the jet nebuliser, which generates aerosols from the liquid drug using a source of compressed gas. Although it is relatively inexpensive, the use jet nebulisers requires long-time treatment, the air compressors are noisy and bulky, and expensive medications are missed in considerable residual volumes.[68]

Alternatively, dry powder inhalers (DPIs) have become highly favourable for delivering high-dose and single-dose drugs with the aid of innovative particle engineering. The

development and use of DPIs have initially been focused on diseases like asthma and COPD. The pulmonary route for these applications offers direct access to the site of action and leads to a rapid onset of action. This decreases the total dose to be given substantially compared to oral administration.[69]

Advantages of DPIs compared with other ways of aerosolization include stable formulations, low cost, automatic coordination between inhalation and dose delivery, and a wide variety of design attributes that can be used to enhance performance.[70] However, drug delivery to the lungs from DPI is known to be inefficient. Is a much greener alternative over the pMDI in terms of stability and avoidance of ozone-depleting and global warming propellants,[66] but its delivery efficiency is not high. Therefore, there is a growing need to enhance and improve the performance of today's devices in order to increase the drug that reaches the lungs, from both an efficiency and cost-containment point-of-view. Examples of different inhaler can be seen in Figure 2.1.

Figure 2.1 Examples of pressurised metered dose inhaler (pMDI), nebuliser and dry powder inhaler (DPIs), respectively.[71–73]

2.1.2. Dry powder inhalers

Dry powder inhaler (DPI) formulations are typically binary mixtures comprising a micronized active agent and a carrier particle population that constitutes the bulk of the formulation (>95% w/w). When a patient inhales through a DPI, the energy resulting from their inspiratory effort fluidizes the powdered dose, detaching a fraction of the drug from the larger carrier particles and depositing it in the deep lung where it applies its therapeutic effect.[74] In DPI, the formulation is placed between the inlet and outlet of an air passage and air is inspired. Difficulties can arise in producing sufficient air flow through the device to entrain the drug and carry it as far as possible into the patients' lung. Air turbulence created through the device and oral cavity dictates, in part, the amount of drug that reaches the lower airways.

In evaluation of existing or future DPI devices, the following key factors should be considered to ensure ideal dose delivery: the device's internal resistance, uniformity of the emitted dose, and aerodynamic particle size distribution.[75] The higher the resistance, the harder it is to generate inspiratory flow great enough to achieve the maximum dose from the inhaler. An ideal DPI would have a low internal resistance and deliver a high respirable fraction, even at low inspiratory flow.[76]

The aim of the current study was to compare four different inhalers' prototypes with different needle's diameters, and evaluate the device resistance and emitted dose of lactose and Foradil.

2.1.3. Reduction of powder retention

Capsule-based DPIs can be limited by powder retention in the capsule and device, which leads to a reduction in the emitted dose. Although the fraction of drug remaining in the mouthpiece is generally between13–20% (determined via in vitro aerosolization tests), in some inhalers, it could be nearly 30–50%.[77] This can be due to mechanical impaction of the powder on the device surface and/or electrostatic charge created during powder dispersion. As pharmaceutical solids and most DPI devices are insulative, the generated electrostatic charge is difficult to dissipate.[78] Therefore, modification of device surface characteristics may successfully reduce the device retention by reducing attractive forces.

Pharmaceutical lubricants like magnesium stearate (MgSt) and leucine have a low surface energy, which can minimize adhesion and friction between particle–particle or particle–inhaler surface.[79] MgSt is an extensively used lubricant for tabletting, and is an approved excipient for inhalation products by the US FDA. Given its recognised safety profiles, coating of MgSt and leucine on inhalable particles has been studied extensively to improve device retention and aerosolisation by reducing particle surface energy and powder cohesion.[80–82] In recent years, a new alternative approach of coating the internal surfaces of capsules and DPI devices with MgSt, to reduce drug retention have been proposed. For the Aerolizer, coating of capsules and inhalers with 0.3 mg/mL of MgSt, significantly reduced capsule and inhaler retentions by 79 and 69%, respectively, compared to the uncoated counterparts. The Rotahaler also showed a considerable reduction in capsule (by 73%) and device retention (by 53%). For both inhalers, reduced capsule and device retentions lead to a significantly higher fine particle doses.[83]

In another report, the surface of a newly designed CC-3D resin inhaler and HPMC capsules were coated with a low surface energy polytetrafluoroethylene suspension.[84]

The capsule, capsule chamber and mouthpiece retention were considerably reduced from 14.1 to 7.3%, 8.8 to 2.2% and 13.2 to 9.1%, respectively.
These studies have shown the efficacy of surface coating to reduce powder retention in capsule and inhaler devices.
In this study, in the inhaler with best performance, with the results of the device resistance and emitted dose, was coated with a force control agent in order to reduce the powder retention in the inhaler's nozzle.

2.1.4. $ScCO_2$-assisted drying

Supercritical drying is a process where the liquid in a substance is transformed into gas in the absence of surface tension and capillary stress. The unique solvent tuneability of supercritical fluids, from gas-like to liquid-like properties, offers the interesting possibility of precise control over the processing conditions, leading to new applications. The use of supercritical fluids technology in the drying process of the developed materials can lead to highly dried and pure products, due to the high diffusivity of CO_2, the fluid phase can easily reach the bulk of the structure. The $scCO_2$ also could be used to dry the wetted materials by selectively extracting the solvent conducing to a thin film of the coating material. Due to the high solubility of ethanol in carbon dioxide, drying of the materials can be successfully obtained.[85]

2.2. Experimental

2.2.1. Materials

DPIs were manufactured by BeyonDevices. Four different DPIs with different needle's diameters of 1.25, 1.5, 1.5 pencil and 2 mm (Figure 2.2) were used. Magnesium stearate was purchased from Sigma-Aldrich. Lactose with two different particle sizes (MM50 and MM3) was purchased from Micro-Sphere S.A.

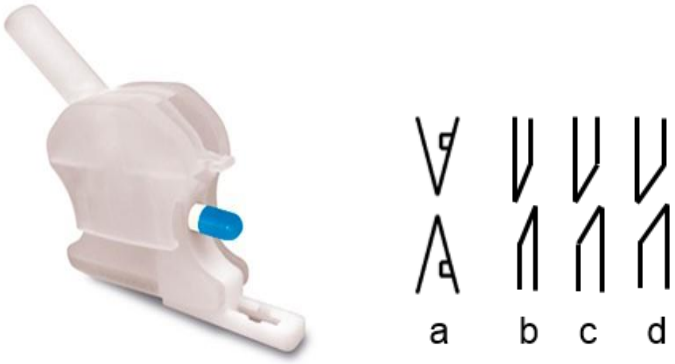


Figure 2.2 BeyonDevices' Inhaler[86] *and schematic representation of different needle's diameters (a) 1.5 pencil, (b) 1.25, (c) 1.5 and (d) 2 mm.*

2.2.2. Characterization of the different Dry Powder Inhalers

2.2.2.1. Experimental apparatus

Figure 2.3 shows a schematic representation of the DUSA apparatus, used for the flux tests.

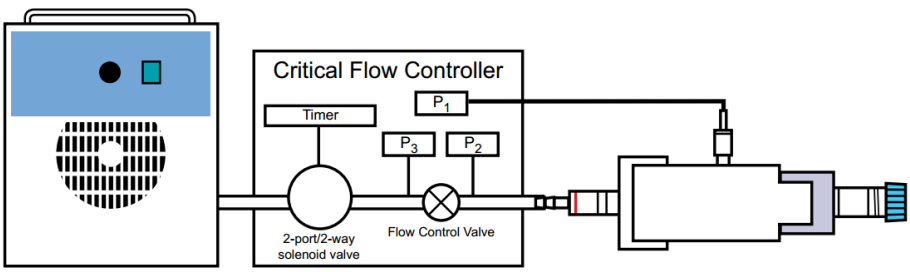


Figure 2.3 Schematic representation of the DUSA apparatus, adapted from Copley Scientific.[87]

2.2.2.2. Flux tests

The flow rates of all four devices were obtained using a Copley Dose Uniformity Sampling Apparatus (DUSA, Copley Scientific), Copley critical flow controller TPK (Copley Scientific), and vacuum pump (Copley Scientific). For this assembly, the DUSA with the mouthpiece adapter was connected to the TPK, and a vacuum source. The pressure measurement port on the DUSA was connected to the TPK to enable measurement of the device flow rate at a defined pressure. An empty capsule was inserted into the device, and the pressure was adjusted to achieve pressures between 1 and 4 KPa. In this way, the flow rate was determined disconnecting the mouthpiece adapter and connecting a flow meter. The specific resistance (R) was then calculated from the flow (Q) and the pressure drop (∆P) using the equation:

$$(\Delta P)^{0.5} = Q.R \qquad \text{eq. 5}$$

2.2.2.3. Uniformity of delivered dose

For delivered dose uniformity testing, the apparatus setup presented in Figure 2.3 was used. Delivered dose uniformity experiments were carried out at 4 KPa for each DPI.[88] Loaded capsules, with 0.080 g of lactose, were placed in a DPI and adapted in a mouthpiece adaptor. The vacuum pump was turn on during a specific time calculated according with the flow rate of inhaler using the equation 6. The emitted dose was calculated by the difference between the weight of inhaler and capsule before and after the assay. Tests were performed for the two different lactoses and in each assay 10 capsules were used.

$$t(s) = \frac{4\ (L)*60\ (s)}{Caudal\ (L/min)} \qquad \text{eq. 6}$$

2.2.3. Modification of Dry Power Inhaler

2.2.3.1. Coating with magnesium stearate

The inhaler was coated with ethanol-based magnesium stearate suspension (0.3 g/mL) via painting and left to dry at room temperature during 2 h.

2.2.3.2. Drying of DPI with $scCO_2$

The drying of coated inhaler was performed in a high-pressure cell with a flow of fresh $scCO_2$ during 2 h at 40°C and 10 MPa.

2.3. Results and discussion

2.3.1. Different Dry Powder Inhalers

2.3.1.1. Flux tests

In order to evaluate which inhaler had the best performance in coating and reducing the powder retention, flux and uniformity of delivered dose tests were performed.
The emitted dose from a DPI can be inhalation flow dependent so information about the resistance of these devices is necessary. Typically, significant differences in the specific resistance of test and reference DPIs may lead to significant differences in drug delivery performance.[89] The internal "force" required for fluidization and disaggregation is dependent on the resistance of the DPI device and the patient's inspiratory effort.[90]

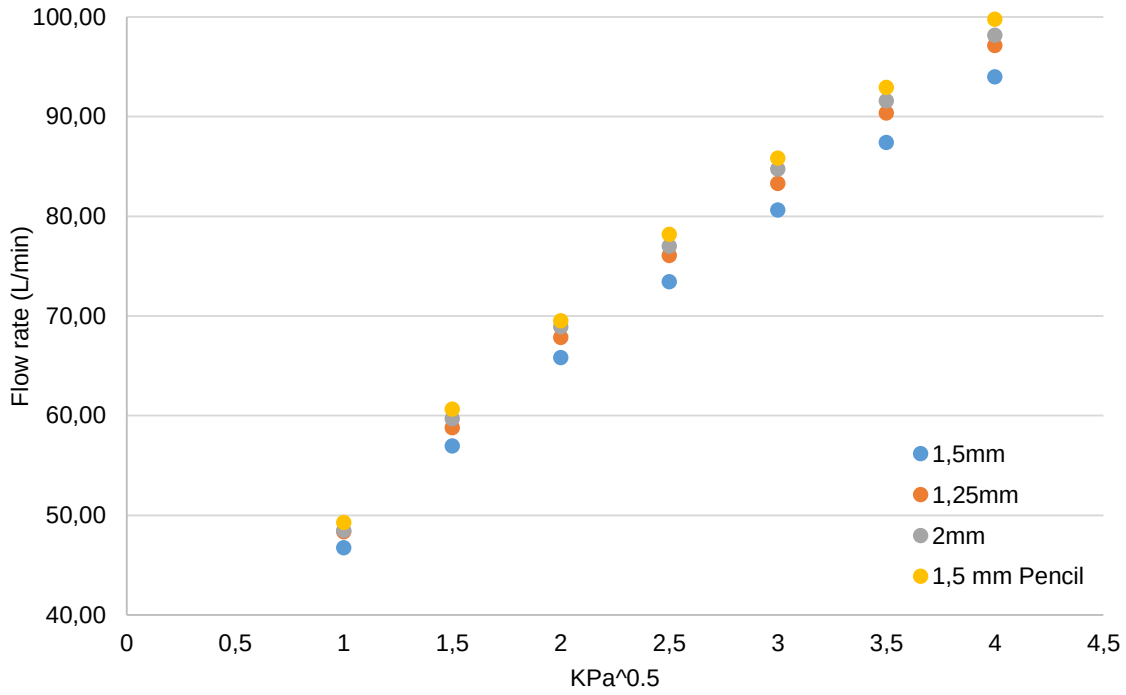


Figure 2.4 Relationship between pressure and flow rate for four different DPIs.

There was almost no difference between the flows through the four different inhalation devices at the different pressure drops (see Figure 2.4). From these measured flows, a mean device resistance of 0.06 $cmH_2O^{0.5}$/(L/min) for the inhalers was obtained.
As shown in the present study, all inhalers can be regarded as low-resistance devices, with values at the lower end of the range observed with most devices, and inspiratory flows of >60 L/min are typically achieved by more than 90% of asthmatic patients.[91]

Devices with high internal resistance may be problematic for patients with severe airway obstruction (i.e., acute asthma or COPD), children, and the elderly, who may have difficulty in exerting sufficient inspiratory effort.

According with a previous study,[90] in the case of 'maximum' effort the pressure drop developed by the chest is constant and that the low device pressure drops at resistances values less than 0.05 $cmH_2O^{0.5}$/(L/min) are the result of an increasing portion of the pressure drop being deployed across the airways. At high resistance the inhaled flow rate will be low and the powder device can afford to generate a slightly coarser aerosol and still ensure penetration into the conducting and lower airways. However, patient 'comfort' is also an important factor and there was some subjective evidence in the above study, as evidenced by unsolicited comments from the volunteers, that resistances greater than about 0.1 $cmH_2O^{0.5}$/(L/min) were 'uncomfortable'. This is probably due to the increased inhalation time and the need for a longer sustained effort with higher resistances.[92]

2.3.1.2. Uniformity of delivered dose

The first test of delivered dose was made using the inhalers with opposite needles and the two different lactose with different particle's size.

Table 2.1 Emitted dose for the four different inhalers and two different lactoses, with the needles in opposite position.

Inhaler	Emitted dose (%)	
	MM50	MM3
1.25 mm	9.4	4.5
1.5 mm	0.9	3.1
1.5 mm pencil	25.2	1.6
2 mm	51.1	3.7

As we can see by the results (see Table 2.1), the emitted dose was very low in this conditions, especially for the lactose MM3, with largest size, where all needles present an emission in the same order. This result could be explained by the shortest space between two needles in the inhaler and also by the humidity present in the lactose MM3, which difficults the exit of powder from the capsule. Due to these results, the following tests were only made for the lactose MM50. Comparing the emission between needles for lactose MM50, the needle with higher diameter showed the best emitted dose, followed by the needle with 1.5 mm pencil. The lowest emission is obtained using a

needle with 1.5 mm, however it was expected that the lowest result would be obtained for the needle with 1.25 mm, since the area to powder crossing was smaller. This result could be explained by the spacing between the in and out needles, that is a little bit smaller for needle with 1.5 mm. Besides this, there is always an amount of powder that is lost in the inhaler nozzle's.

Table 2.2 Emitted dose for the four different inhalers and lactose MM50, with the needles in same position.

Inhaler	Emitted dose (%) MM50
1.25 mm	24.9
1.5 mm	7.5
1.5 mm pencil	20.3
2 mm	68.9

By changing the orientation of the needles (Table 2.2), it was possible to observe an increase in the emitted dose for all different inhalers, with exception for the inhaler with a pencil needle. This kind of needle presents a lateral hole in contrast with the other ones that present the hole at the extremity (see Figure 2.2). By this way, it is possible to confirm that the air flow is more effective, leading to a better transportation of the powder. As expected, the needle with highest diameter presents best results, with an emitted dose of 68.9%. Thus, this was the inhaler chosen for the tests with a real formulation.

Foradil consists of a dry powder formulation of formoterol fumarate intended for oral inhalation with an inhaler. The inhalation powder is packaged in clear hard gelatin capsules. Each capsule contains a dry powder blend of 12 µg of formoterol fumarate and 25 mg of lactose as a carrier. This formulation was used as model formulation in the uniformity emitted dose tests.

Table 2.3 Emitted dose for the inhaler with needle diameter's of 2 mm and Foradil, with the needles in same position.

Inhaler	Emitted dose (%)
2 mm	30.0

The emitted dose (Table 2.3) obtained for this formulation using the inhaler with 2 mm needle was lower than obtained for lactose. This result could be explained by the amount of powder in the capsule, which was 0.080 g for lactose and 0.025 g for Foradil,

confirming the smaller amount of powder available to be emitted, and also the smaller amount present between the two needles, where the air pass and drag the powder.
The emitted dose from the Foradil obtained in this study is lower than previous reported studies (82%[93] and 90%[94] at flows of 60 L/min).

2.3.2. Comparison of optimized device performance

2.3.2.1. Powder retention

MgSt is a commonly-used force control agent and was applied to the inside surface of the inhaler nozzle's to minimize powder retention and increase the fraction entering the lungs.

Table 2.4 Emitted dose for the inhaler with needle diameter's of 2 mm, with the inside surface of the inhaler nozzle's coated with MgSt, using lactose MM50 and the needles in same position.

Inhaler	Emitted dose (%)
2 mm	71.3

Table 2.4 shows the emitted dose for the inhaler with the inside surface nozzle's coated with MgSt and needle diameter's of 2 mm, using lactose MM50 and the needles in same position. As can be seen, the powder retention decreased. This could be due to the reduced adhesion between the powder and the inhaler wall which brought amount much improved flow properties.[95]

Table 2.5 Emitted dose for the inhaler with needle diameter's of 2 mm, with the inside surface of the inhaler nozzle's coated with MgSt, using Foradil and the needles in same position.

Inhaler	Emitted dose (%)
2 mm	33,7

Using the same idea, the emitted dose for Foradil also increases (Table 2.5), as expected. In order to further increase the emitted dose, the capsule could also be coated with MgSt, to reduce the powder that stays in the capsule walls. However, this value is still lower than that obtained in other reported studies in literature for this formulation using other inhalers.[94]

2.4. Conclusion

In order to evaluate the performance of dry powder inhalers with different needles, internal resistance and emitted dose with lactose was tested. The internal device resistance was similar for all inhalers and the mean value was 0.06 $cmH_2O^{0.5}$/(L/min), regarding the inhalers as low-resistance devices with inspiratory flow above 60 L/min. The best results for emitted dose were observed for needles in the same orientation, and the higher emitted dose was obtained using a 2 mm needle, the higher diameter tested, where 68.9% of the lactose in the capsule was emitted.

A coating of inhaler's nozzle with pharmaceutically acceptable force-control agent to reduce drug retention was developed in the previous best inhaler. Magnesium stearate was painted in the internal surface of the inhaler and dried with supercritical carbon dioxide in order to remove all solvent.This coating minimized the adhesion between the drug particles and the internal surfaces of the DPI, which increased the emitted dose by 2.4% for lactose and 3.7% for Foradil. The increase in inhaled dose result in enhanced therapeutic benefits, and in reduction of production coasts.

Future uptake of this technology by industry could be via direct incorporation of the surface modifier into the device manufacturing process.

The tested inhalers need some improvements in order to increase the emitted dose, such as changes in the internal design, reduction in the air inlets or change the design of needles, since the powder has some difficult in through the needles and reach the nozzle.

Final conclusions

The development of a master thesis in collaboration with a company is an important feature in the improvement of the ability to analyze problems from a research perspective keeping in mind the constraints and limitations of the real industry, realizing what creates a real value for the company.

The present work has successfully demonstrated the possibility of improving devices from the real market using the academic knowledge and technologies available in the host laboratory.

As future goals, it will be interesting to test the direct application of these modifications in the manufacturing process of the devices. Investigation of other kind of modifications that confer additional properties could be an additional useful feature of such medical devices. Regarding the vaginal applicator and inhaler, the improvement of the sliding factor, the mucoadhesion ability, the drug residence time or powder flowability are some examples of promising studies that could bring further value to the devices.

References

(1) Klevens, R. M.; Edwards, J. R.; Richards, C. L.; Horan, T. C.; Gaynes, R. P.; Pollock, D. A.; Cardo, D. M. Estimating Health Care-Associated Infections and Deaths in U.S. Hospitals. *Public Health Rep.* **2002**, *122*, 160–166.

(2) Von Eiff, C.; Jansen, B.; Kohnen, W.; Becker, K. Infections Associated with Medical Devices. *Drugs* **2005**, *65*, 179–214.

(3) Dickinson, G. M.; Bisno, A. L. Infections Associated with Indwelling Devices: Infections Related to Extravascular Devices. *Antimicrob. Agents Chemother.* **1989**, *33*, 602–607.

(4) Peters, G.; Locci, R.; Pulverer, G. Adherence and Growth of Coagulase-Negative Staphylococci on Surfaces of Intravenous Catheters. *J. Infect. Dis.* **1982**, *146*, 479–482.

(5) Weber, D. J.; Rutala, W. A. Self-Disinfecting Surfaces: Review of Current Methodologies and Future Prospects. *Am. J. Infect. Control* **2013**, *41*, S31–5.

(6) BeyonDevices unique Vaginal Applicator http://www.beyondevices.eu/index.php/women-s-health/2013-12-17-16-15-21 (accessed Jul 15, 2014).

(7) Sobel, J. D. Pathogenesis of Recurrent Vulvovaginal Candidiasis. *Curr. Infect. Dis. Rep.* **2002**, *4*, 514–519.

(8) Schaeffer, Anthony J.; Joanne M. Jones, J. K. D. Association of in Vitro Escherichia Coli Adherence to Vaginal and Buccal Epithelial Cells with Susceptibility of Women to Recurrent Urinary-Tract Infections. *N. Engl. J. Med.* **1981**, 1062–1066.

(9) Bazaka, K.; Jacob, M. V; Crawford, R. J.; Ivanova, E. P. Plasma-Assisted Surface Modification of Organic Biopolymers to Prevent Bacterial Attachment. *Acta Biomater.* **2011**, *7*, 2015–2028.

(10) Mussard, W.; Kebir, N.; Kriegel, I.; Estève, M.; Semetey, V. Facile and Efficient Control of Bioadhesion on Poly(dimethylsiloxane) by Using a Biomimetic Approach. *Angew. Chem. Int. Ed. Engl.* **2011**, *50*, 10871–10874.

(11) Alexander, J. W. History of the Medical Use of Silver. *Surg. Infect. (Larchmt).* **2009**, *10*, 289–292.

(12) Grass, G.; Rensing, C.; Solioz, M. Metallic Copper as an Antimicrobial Surface. *Appl. Environ. Microbiol.* **2011**, *77*, 1541–1547.

(13) Campoccia, D.; Montanaro, L.; Speziale, P.; Arciola, C. R. Antibiotic-Loaded Biomaterials and the Risks for the Spread of Antibiotic Resistance Following Their Prophylactic and Therapeutic Clinical Use. *Biomaterials* **2010**, *31*, 6363–6377.

(14) Hancock, R. E. W.; Sahl, H.-G. Antimicrobial and Host-Defense Peptides as New Anti-Infective Therapeutic Strategies. *Nat. Biotechnol.* **2006**, *24*, 1551–1557.

(15) De la Fuente-Núñez, C.; Korolik, V.; Bains, M.; Nguyen, U.; Breidenstein, E. B. M.; Horsman, S.; Lewenza, S.; Burrows, L.; Hancock, R. E. W. Inhibition of Bacterial Biofilm Formation and Swarming Motility by a Small Synthetic Cationic Peptide. *Antimicrob. Agents Chemother.* **2012**, *56*, 2696–2704.

(16) Campoccia, D.; Montanaro, L.; Arciola, C. R. A Review of the Biomaterials Technologies for Infection-Resistant Surfaces. *Biomaterials* **2013**, *34*, 8533–8554.

(17) Knetsch, M. L. W.; Koole, L. H. New Strategies in the Development of Antimicrobial Coatings: The Example of Increasing Usage of Silver and Silver Nanoparticles. *Polymers (Basel).* **2011**, *3*, 340–366.

(18) Siedenbiedel, F.; Tiller, J. C. Antimicrobial Polymers in Solution and on Surfaces: Overview and Functional Principles. *Polymers (Basel).* **2012**, *4*, 46–71.

(19) Fik, C. P.; Krumm, C.; Muennig, C.; Baur, T. I.; Salz, U.; Bock, T.; Tiller, J. C. Impact of Functional Satellite Groups on the Antimicrobial Activity and Hemocompatibility of Telechelic poly(2-Methyloxazoline)s. *Biomacromolecules* **2012**, *13*, 165–172.

(20) Qiao, Y.; Yang, C.; Coady, D. J.; Ong, Z. Y.; Hedrick, J. L.; Yang, Y.-Y. Highly Dynamic Biodegradable Micelles Capable of Lysing Gram-Positive and Gram-Negative Bacterial Membrane. *Biomaterials* **2012**, *33*, 1146–1153.

(21) Mizutani, M.; Palermo, E. F.; Thoma, L. M.; Satoh, K.; Kamigaito, M.; Kuroda, K. Design and Synthesis of Self-Degradable Antibacterial Polymers by Simultaneous Chain- and Step-Growth Radical Copolymerization. *Biomacromolecules* **2012**, *13*, 1554–1563.

(22) Waschinski, C. J.; Tiller, J. C. Poly(oxazoline)s with Telechelic Antimicrobial Functions. *Biomacromolecules* **2004**, *6*, 235–243.

(23) Adams, N.; Schubert, U. S. Poly(2-Oxazolines) in Biological and Biomedical Application Contexts. *Adv. Drug Deliv. Rev.* **2007**, *59*, 1504–1520.

(24) Konradi, R.; Acikgoz, C.; Textor, M. Polyoxazolines for Nonfouling Surface Coatings--a Direct Comparison to the Gold Standard PEG. *Macromol. Rapid Commun.* **2012**, *33*, 1663–1676.

(25) Jevsevar, S.; Kunstelj, M.; Porekar, V. G. PEGylation of Therapeutic Proteins. *Biotechnol. J.* **2010**, *5*, 113–128.

(26) Arima, Y.; Toda, M.; Iwata, H. Complement Activation on Surfaces Modified with Ethylene Glycol Units. *Biomaterials* **2008**, *29*, 551–560.

(27) Mero, A.; Pasut, G.; Dalla Via, L.; Fijten, M. W. M.; Schubert, U. S.; Hoogenboom, R.; Veronese, F. M. Synthesis and Characterization of poly(2-Ethyl 2-Oxazoline)-Conjugates with Proteins and Drugs: Suitable Alternatives to PEG-Conjugates? *J. Control. Release* **2008**, *125*, 87–95.

(28) Sedlacek, O.; Monnery, B. D.; Filippov, S. K.; Hoogenboom, R.; Hruby, M. Poly(2-Oxazoline)s--Are They More Advantageous for Biomedical Applications than Other Polymers? *Macromol. Rapid Commun.* **2012**, *33*, 1648–1662.

(29) De Macedo, C. V.; da Silva, M. S.; Casimiro, T.; Cabrita, E. J.; Aguiar-Ricardo, A. Boron Trifluoride Catalyzed Polymerisation of 2-Substituted-2-Oxazolines in Supercritical Carbon Dioxide. *Green Chem.* **2007**, *9*, 948.

(30) Correia, V. G.; Bonifácio, V. D. B.; Raje, V. P.; Casimiro, T.; Moutinho, G.; da Silva, C. L.; Pinho, M. G.; Aguiar-Ricardo, A. Oxazoline-Based Antimicrobial Oligomers: Synthesis by CROP Using Supercritical CO2. *Macromol. Biosci.* **2011**, *11*, 1128–1137.

(31) Waschinski, C. J.; Herdes, V.; Schueler, F.; Tiller, J. C. Influence of Satellite Groups on Telechelic Antimicrobial Functions of Polyoxazolines. *Macromol. Biosci.* **2005**, *5*, 149–156.

(32) Tiller, J. C.; Liao, C. J.; Lewis, K.; Klibanov, A. M. Designing Surfaces That Kill Bacteria on Contact. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 5981–5985.

(33) Reverchon, E.; Porta, G. Della; Senatore, F. Supercritical CO2 Extraction and Fractionation of Lavender Essential Oil and Waxes. *J. Agric. Food Chem.* **1995**, *43*, 1654–1658.

(34) Hammer, K. A.; Carson, C. F.; Riley, T. V. Antimicrobial Activity of Essential Oils and Other Plant Extracts. *J. Appl. Microbiol.* **1999**, *86*, 985–990.

(35) Nelson, R. R. In-Vitro Activities of Five Plant Essential Oils against Methicillin-Resistant Staphylococcus Aureus and Vancomycin-Resistant Enterococcus Faecium. *J. Antimicrob. Chemother.* **1997**, 305–306.

(36) Deans, S. G.; Ritchie, G. Antibacterial Properties of Plant Essential Oils. *Int. J. Food Microbiol.* **1987**, *5*, 165–180.

(37) Eckert, C. A.; Knutson, B. L.; Debenedetti, P. G. Supercritical Fluids as Solvents for Chemical and Materials Processing. *Nature* **1996**, *383*, 313–318.

(38) Jessop, P. G.; Leitner, W. *Chemical Synthesis Using Supercritical Fluids*; Wiley-VCH, 1999.

(39) Center for Scientific Creation
http://www.creationscience.com/onlinebook/HydroplateOverview9.html
(accessed Jul 26, 2014).

(40) GeoScienceWorld
http://elements.geoscienceworld.org/content/3/3/179/F2.expansion.html
(accessed Aug 14, 2014).

(41) Cooper, A. I.; DeSimone, J. M. Polymer Synthesis and Characterization in Liquid / Supercritical Carbon Dioxide. *Curr. Opin. Solid State Mater. Sci.* **1996**, *1*, 761–768.

(42) Reverchon, E. Supercritical Fluid Extraction and Fractionation of Essential Oils and Related Products. *J. Supercrit. Fluids* **1997**, *10*, 1–37.

(43) Shim, J.-J.; Johnston, K. P. Molecular Thermodynamics of Solute-Polymer-Supercritical Fluid Systems. *AIChE J.* **1991**, *37*, 607–616.

(44) Dohrn, R.; Fonseca, J. M. S.; Peper, S. Experimental Methods for Phase Equilibria at High Pressures. *Annu. Rev. Chem. Biomol. Eng.* **2012**, *3*, 343–367.

(45) Watkins, J. J.; McCarthy, T. J. Polymerization in Supercritical Fluid-Swollen Polymers: A New Route to Polymer Blends. *Macromolecules* **1994**, *27*, 4845–4847.

(46) Ma, X.; Tomasko, D. L. Coating and Impregnation of a Nonwoven Fibrous Polyethylene Material with a Nonionic Surfactant Using Supercritical Carbon Dioxide. *Ind. Eng. Chem. Res.* **1997**, *36*, 1586–1597.

(47) Berens, A. R.; Huvard, G. S.; Korsmeyer, R. W.; Kunig, F. W. Application of Compressed Carbon Dioxide in the Incorporation of Additives into Polymers. *J. Appl. Polym. Sci.* **1992**, *46*, 231–242.

(48) Lobato, C.; Pinho, M. G.; Aguiar-ricardo, A. Oxazoline-Based Antimicrobial Oligomers : Synthesis by CROP Using. **2011**, 1–10.

(49) Ribeiro, M. P.; Espiga, A.; Silva, D.; Baptista, P.; Henriques, J.; Ferreira, C.; Silva, J. C.; Borges, J. P.; Pires, E.; Chaves, P.; Correia, I. J. Development of a New Chitosan Hydrogel for Wound Dressing. *Wound Repair Regen.* **2009**, *17*, 817–824.

(50) Ribeiro, M. P.; Morgado, P. I.; Miguel, S. P.; Coutinho, P.; Correia, I. J. Dextran-Based Hydrogel Containing Chitosan Microparticles Loaded with Growth Factors to Be Used in Wound Healing. *Mater. Sci. Eng. C. Mater. Biol. Appl.* **2013**, *33*, 2958–2966.

(51) Morgado, P. I.; Lisboa, P. F.; Ribeiro, M. P.; Miguel, S. P.; Simões, P. C.; Correia, I. J.; Aguiar-Ricardo, A. Poly(vinyl Alcohol)/chitosan Asymmetric Membranes: Highly Controlled Morphology toward the Ideal Wound Dressing. *J. Memb. Sci.* **2014**, *469*, 262–271.

(52) Gonzalez, R. .; Tarloff, J. . Evaluation of Hepatic Subcellular Fractions for Alamar Blue and MTT Reductase Activity. *Toxicol. Vitr.* **2001**, *15*, 257–259.

(53) Palmeira-de-Oliveira, A.; Ribeiro, M. P.; Palmeira-de-Oliveira, R.; Gaspar, C.; Costa-de-Oliveira, S.; Correia, I. J.; Pina Vaz, C.; Martinez-de-Oliveira, J.; Queiroz, J. A.; Rodrigues, A. G. Anti-Candida Activity of a Chitosan Hydrogel: Mechanism of Action and Cytotoxicity Profile. *Gynecol. Obstet. Invest.* **2010**, *70*, 322–327.

(54) Busby, A. J.; Zhang, J.; Naylor, A.; Roberts, C. J.; Davies, M. C.; Tendler, S. J. B.; Howdle, S. M. The Preparation of Novel Nano-Structured Polymer Blends of Ultra High Molecular Weight Polyethylene with Polymethacrylates Using Supercritical Carbon Dioxide. *J. Mater. Chem.* **2003**, *13*, 2838.

(55) D'Auria, F. D.; Tecca, M.; Strippoli, V.; Salvatore, G.; Battinelli, L.; Mazzanti, G. Antifungal Activity of Lavandula Angustifolia Essential Oil against Candida Albicans Yeast and Mycelial Form. *Med. Mycol.* **2005**, *43*, 391–396.

(56) Cavanagh, H. M. A.; Wilkinson, J. M. Biological Activities of Lavender Essential Oil. *Phytother. Res.* **2002**, *16*, 301–308.

(57) Lin, J.; Tiller, J. C.; Lee, S. B.; Lewis, K.; Klibanov, A. M. Insights into Bactericidal Action of Surface-Attached Poly(vinyl-N-Hexylpyridinium) Chains. *Biotechnol. Lett.* **2002**, *24*, 801–805.

(58) Ha, J.-U.; Kim, Y.-M.; Lee, D.-S. Multilayered Antimicrobial Polyethylene Films Applied to the Packaging of Ground Beef. *Packag. Technol. Sci.* **2001**, *14*, 55–62.

(59) Correia, V. G.; Coelho, M.; Barroso, T.; Raje, V. P.; Bonifácio, V. D. B.; Casimiro, T.; Pinho, M. G.; Aguiar-Ricardo, A. Anti-Biofouling 3D Porous Systems: The Blend Effect of Oxazoline-Based Oligomers on Chitosan Scaffolds. *Biofouling* **2013**, *29*, 273–282.

(60) Contreras-García, A.; Bucio, E.; Brackman, G.; Coenye, T.; Concheiro, A.; Alvarez-Lorenzo, C. Biofilm Inhibition and Drug-Eluting Properties of Novel DMAEMA-Modified Polyethylene and Silicone Rubber Surfaces. *Biofouling* **2011**, *27*, 123–135.

(61) Waschinski, C. J.; Herdes, V.; Schueler, F.; Tiller, J. C. Influence of Satellite Groups on Telechelic Antimicrobial Functions of Polyoxazolines. *Macromol. Biosci.* **2005**, *5*, 149–156.

(62) Waschinski, C. J.; Barnert, S.; Theobald, A.; Schubert, R.; Kleinschmidt, F.; Hoffmann, A.; Saalwächter, K.; Tiller, J. C. Insights in the Antibacterial Action of Poly(methyloxazoline)s with a Biocidal End Group and Varying Satellite Groups. *Biomacromolecules* **2008**, *9*, 1764–1771.

(63) Anderson, B. C.; Bloom, P. D.; Baikerikar, K. G.; Sheares, V. V.; Mallapragada, S. K. Al–Cu–Fe Quasicrystal/ultra-High Molecular Weight Polyethylene Composites as Biomaterials for Acetabular Cup Prosthetics. *Biomaterials* **2002**, *23*, 1761–1768.

(64) Kaparissides, C.; Alexandridou, S.; Kotti, K.; Chaitidou, S. Recent Advances in Novel Drug Delivery Systems. *J. Nanotechnol. Online 2* **2006**, 1–11.

(65) Coates, M. S.; Fletcher, D. F.; Chan, H.-K.; Raper, J. A. Effect of Design on the Performance of a Dry Powder Inhaler Using Computational Fluid Dynamics. Part 1: Grid Structure and Mouthpiece Length. *J. Pharm. Sci.* **2004**, *93*, 2863–2876.

(66) Leach, C. L. The CFC to HFA Transition and Its Impact on Pulmonary Drug Development. *Respir Care* **2005**, *50*, 1201–1208.

(67) Patton; S., J. Pulmonary Drug Delivery Comes of Age: The Outlook for 2005 & Beyond. *Drug Deliv. Technol.* **2005**, 6–9.

(68) Gonda, I. The Ascent of Pulmonary Drug Delivery. *J. Pharm. Sci.* **2000**, *89*, 940–945.

(69) Labiris, N. R.; Dolovich, M. B. Pulmonary Drug Delivery. Part I: Physiological Factors Affecting Therapeutic Effectiveness of Aerosolized Medications. *Br. J. Clin. Pharmacol.* **2003**, *56*, 588–599.

(70) Traini, D.; Young, P. M. Delivery of Antibiotics to the Respiratory Tract: An Update. *Expert Opin. Drug Deliv.* **2009**, *6*, 897–905.

(71) Monodose Inhaler http://www.miat.it/en/monodose_inhaler.php (accessed Aug 21, 2014).

(72) Nebulizer Machine http://www.onlinesurgicals.com/store/respiratory/nebulizer-machine (accessed Aug 21, 2014).

(73) Inhaler For Asthma http://www.asthmasymptoms86.com/ventolin-inhaler.html#.VAM7SfmwLcg (accessed Aug 21, 2014).

(74) Deboer, A.; Hagedoorn, P.; Gjaltema, D.; Goede, J.; Frijlink, H. Air Classifier Technology (ACT) in Dry Powder inhalationPart 1. Introduction of a Novel Force Distribution Concept (FDC) Explaining the Performance of a Basic Air Classifier on Adhesive Mixtures. *Int. J. Pharm.* **2003**, *260*, 187–200.

(75) Dolovich, M. New Propellant-Free Technologies under Investigation. *J. Aerosol Med.* **1999**, *12*, S–9–S–17.

(76) Ashurst, I.; Malton, A.; Prime, D.; Sumby, B. Latest Advances in the Development of Dry Powder Inhalers. *Pharm. Sci. Technolo. Today* **2000**, *3*, 246–256.

(77) Hu, T.-T.; Zhao, H.; Jiang, L.-C.; Le, Y.; Chen, J.-F.; Yun, J. Engineering Pharmaceutical Fine Particles of Budesonide for Dry Powder Inhalation (DPI). *Ind. Eng. Chem. Res.* **2008**, *47*, 9623–9627.

(78) Karner, S.; Anne Urbanetz, N. The Impact of Electrostatic Charge in Pharmaceutical Powders with Specific Focus on Inhalation-Powders. *J. Aerosol Sci.* **2011**, *42*, 428–445.

(79) Zhou, Q. T.; Morton, D. A. V. Drug-Lactose Binding Aspects in Adhesive Mixtures: Controlling Performance in Dry Powder Inhaler Formulations by Altering Lactose Carrier Surfaces. *Adv. Drug Deliv. Rev.* **2012**, *64*, 275–284.

(80) Zhou, Q. T.; Qu, L.; Gengenbach, T.; Larson, I.; Stewart, P. J.; Morton, D. A. V. Effect of Surface Coating with Magnesium Stearate via Mechanical Dry Powder Coating Approach on the Aerosol Performance of Micronized Drug Powders from Dry Powder Inhalers. *AAPS PharmSciTech* **2013**, *14*, 38–44.

(81) Paajanen, M.; Katainen, J.; Raula, J.; Kauppinen, E. I.; Lahtinen, J. Direct Evidence on Reduced Adhesion of Salbutamol Sulphate Particles due to L-Leucine Coating. *Powder Technol.* **2009**, *192*, 6–11.

(82) Zhou, Q. T.; Denman, J. A.; Gengenbach, T.; Das, S.; Qu, L.; Zhang, H.; Larson, I.; Stewart, P. J.; Morton, D. A. V. Characterization of the Surface Properties of a Model Pharmaceutical Fine Powder Modified with a Pharmaceutical Lubricant to Improve Flow via a Mechanical Dry Coating Approach. *J. Pharm. Sci.* **2011**, *100*, 3421–3430.

(83) Heng, D.; Lee, S. H.; Ng, W. K.; Chan, H.-K.; Kwek, J. W.; Tan, R. B. H. Novel Alternatives to Reduce Powder Retention in the Dry Powder Inhaler during Aerosolization. *Int. J. Pharm.* **2013**, *452*, 194–200.

(84) Behara, S. R. B.; Farkas, D. R.; Hindle, M.; Longest, P. W. Development of a High Efficiency Dry Powder Inhaler: Effects of Capsule Chamber Design and Inhaler Surface Modifications. *Pharm. Res.* **2014**, *31*, 360–372.

(85) Teberikler, L.; Koseoglu, S.; Akgerman, A. Selective Extraction of Phosphatidylcholine from Lecithin by Supercritical Carbon Dioxide/ethanol Mixture. *J. Am. Oil Chem. Soc.* **2001**, *78*, 115–120.

(86) BeyonDevices DPI http://www.beyondevices.eu/index.php/inhalation/2013-12-17-16-17-52 (accessed Aug 23, 2014).

(87) Copley Scientific. Quality Solutions for Inhaler Testing http://www.copleyscientific.com/documents/ww/Inhaler Brochure 2012 (Low Res).pdf (accessed Aug 31, 2014).

(88) Council of Europe. Preparations for Inhalation. *Eur. Pharmacopoeia 5.1. Counc. Eur.* **2004**, 2843–2847.

(89) NEWMAN, S. P.; BUSSE, W. W. Evolution of Dry Powder Inhaler Design, Formulation, and Performance. *Respir. Med.* **2002**, *96*, 293–304.

(90) CLARK, A. R.; HOLLINGWORTH, A. M. The Relationship Between Powder Inhaler Resistance and Peak Inspiratory Conditions in Healthy Volunteers — Implications for In Vitro Testing. *J. Aerosol Med.* **1993**, *6*, 99–110.

(91) Srichana, T.; Martin, G. P.; Marriott, C. Dry Powder Inhalers: The Influence of Device Resistance and Powder Formulation on Drug and Lactose Deposition in Vitro. *Eur. J. Pharm. Sci.* **1998**, *7*, 73–80.

(92) Pedersen, S.; Steffensen, G. Fenoterol Powder Inhaler Technique in Children: Influence of Inspiratory Flow Rate and Breath-Holding. *Eur. J. Respir. Dis.* **1986**, *68*, 207–214.

(93) Weuthen, T.; Roeder, S.; Brand, P.; Müllinger, B.; Scheuch, G. In Vitro Testing of Two Formoterol Dry Powder Inhalers at Different Flow Rates. *J. Aerosol Med.* **2002**, *15*, 297–303.

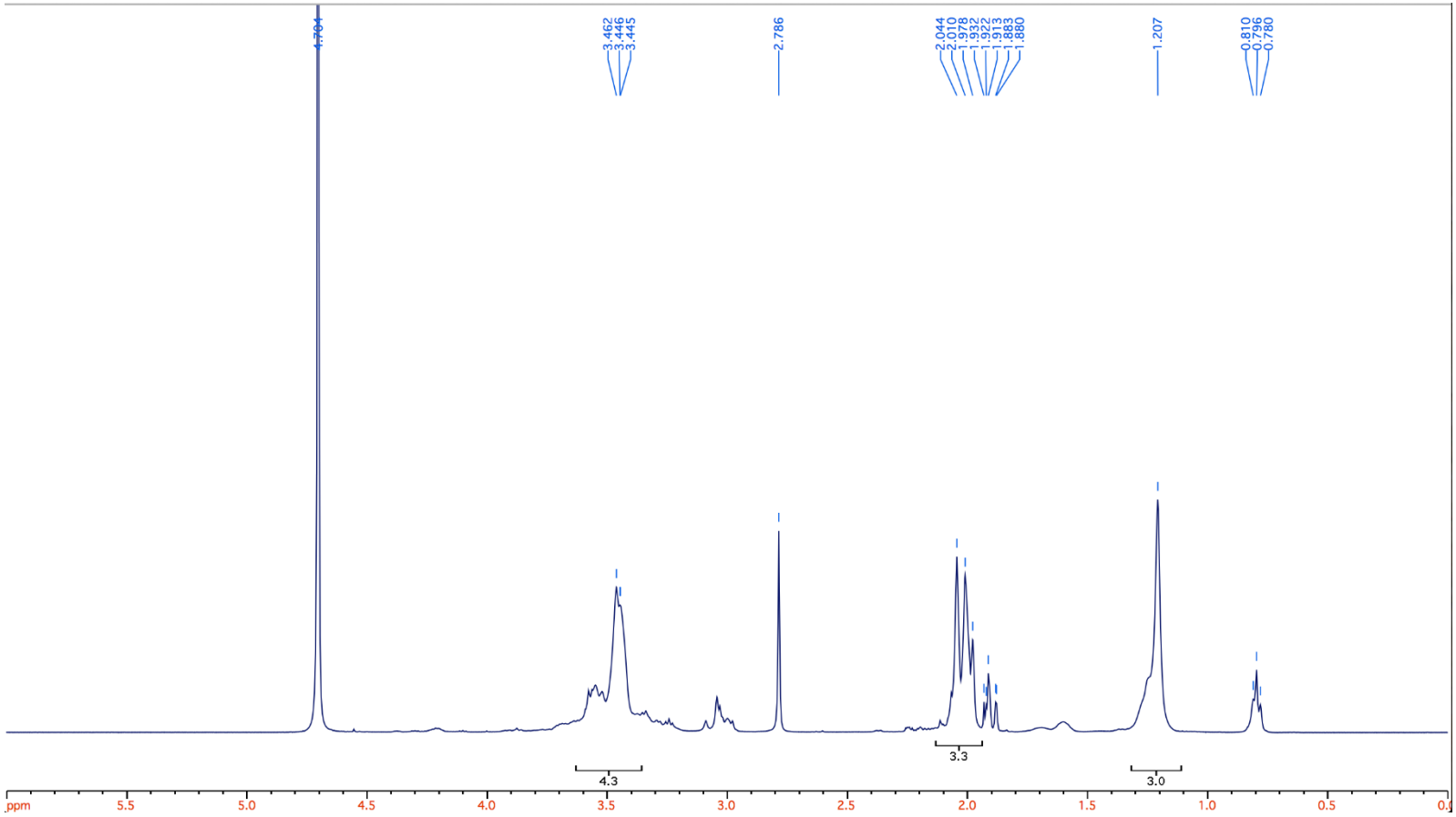
(94) Chew, N. Y.; Chan, H. K. In Vitro Aerosol Performance and Dose Uniformity between the Foradile Aerolizer and the Oxis Turbuhaler. *J. Aerosol Med.* **2001**, *14*, 495–501.

(95) Wang, J.; Wen, H.; Desai, D. Lubrication in Tablet Formulations. *Eur. J. Pharm. Biopharm.* **2010**, *75*, 1–15.

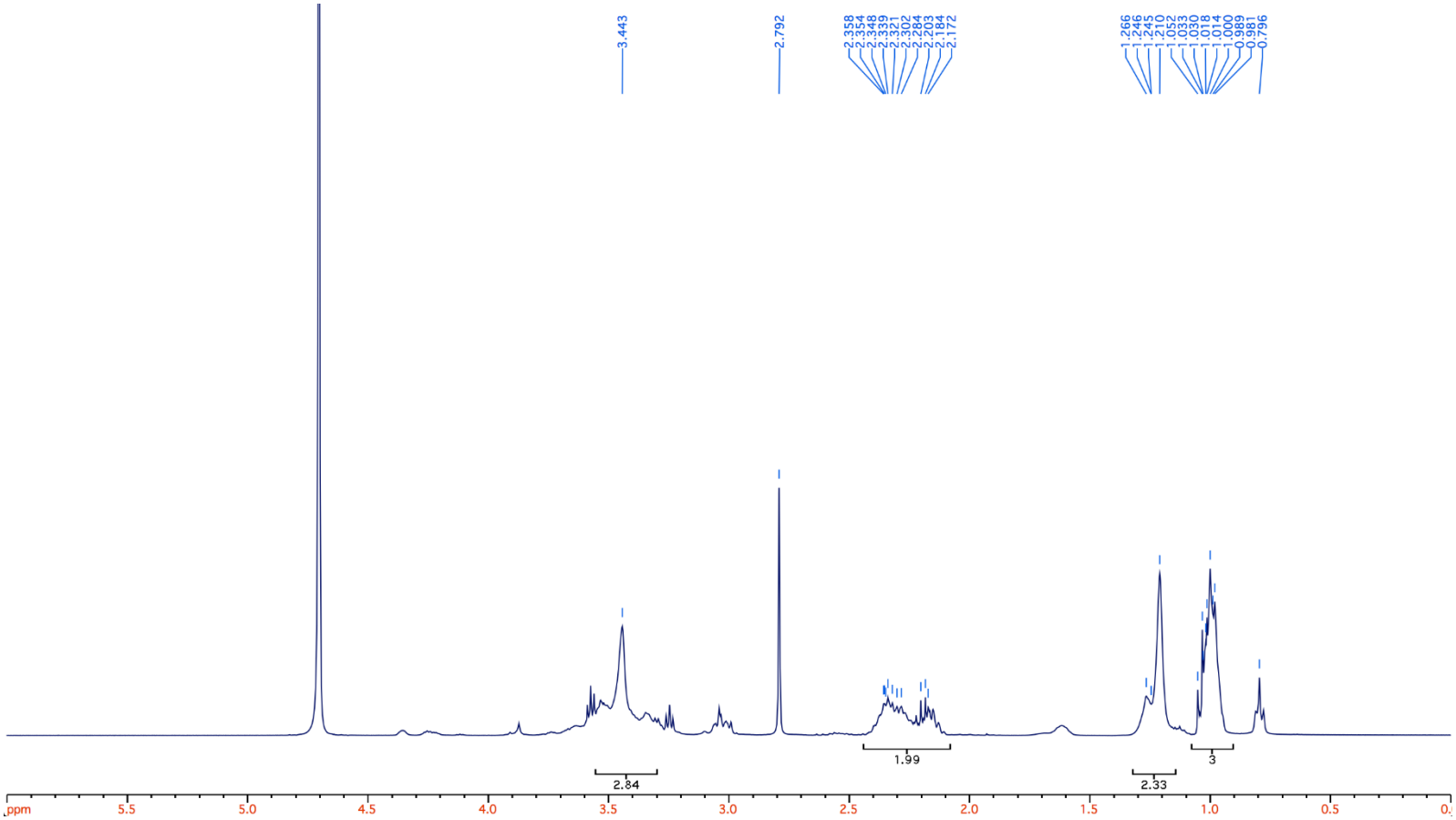
Appendix section

Appendix 1 Composition of Lavender essential oil obtained by $scC0_2$ extraction (% calculated by area integration).

Compound	Composition (%)
Trans-β-ocimene	3.99
Cis-β-ocimene	5.80
α-ocimene	1.28
1-octen-3-yl acetate	2.84
Linalool	15.99
Linalyl acetate	36.02
Farnesene	0.19
β-Caryophyllen	6.10
α-terpeniol	1.17
Lavandulyl acetate	5.86
Germancrene	3.41
Lavandulol	1.00
Fenchol	<0.1
Borneol	0.86



Appendix 2 ^{1}H-NMR spectrum (400 MHz, D_2O) of MeOx.



Appendix 3 ^{1}H-NMR spectrum (400 MHz, D_2O) of EtOx.